# CONNECTIVE TISSUE DISORDERS. DETECTION OF ANTI-NUCLEAR ANTIBODIES AND COMPARATIVE EVALUATION OF ENZYME IMMUNE ASSAY WITH INDIRECT IMMUNO-FLUORESCENCE ASSAY.

**Dissertation submitted in partial fulfillment of the**
**Requirement for the award of the Degree of**

**M. D. MICROBIOLOGY**
**BRANCH -- IV**

**THE TAMIL NADU DR. M. G. R MEDICAL UNIVERSITY**
**CHENNAI- 600032**
**APRIL2016**


**DEPARTMENT OF MICROBIOLOGY**
**TIRUNELVELI MEDICAL COLLEGE**
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## CERTIFICATE

This is to certify that the Dissertation "**CONNECTIVE TISSUE DISORDERS. DETECTION OF ANTI-NUCLEAR ANTIBODIES AND COMPARATIVE EVALUATION OF ENZYME IMMUNE ASSAY WITH INDIRECT IMMUNOFLUORESCENCE ASSAY**" presented herein by **Dr. S.SUGANYA** is an original work done in the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D.( Branch IV ) Microbiology under my guidance and supervision during the academic period of 2013-2016.

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PROTOCOL TITLE: CONNECTIVE TISSUE DISORDER- DETECTION OF ANTINUCLEAR ANTIBODIES & COMPARATIVE EVALUATION OF ENZYME IMMUNE ASSAY WITH INDIRECT IMMUNOFLURESCENCE.

PRINCIPAL INVESTIGATOR: Dr. S.SUGANYA, MBBS.,

DESIGNATION OF PRINCIPAL INVESTIGATOR: POST GRADUATE IN MD MICROBIOLOGY
DEPARTMENT & INSTITUTION: TIRUNELVELI MEDICAL COLLEGE , TIRUNELVELI

*Dear , Dr.S.Suganya, MBBS., The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 09.02.15.*

**THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED**

1. TIREC Application Form ☐
2. Study Protocol ☐
3. Department Research Committee Approval ☐
4. Patient Information Document and Consent Form in English and Vernacular Language ☐
5. Investigator's Brochure ☐
6. Proposed Methods for Patient Accrual Proposed ☐
7. Curriculum Vitae of the Principal Investigator ☐
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10. Investigator's Undertaking ☐
11. DCGI/DGFT approval ☐
12. Clinical Trial Agreement (CTA) ☐
13. Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA) ☐
14. Clinical Trials Registry-India (CTRI) Registration ☐

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1. The approval is valid for a period of 2 year/s or duration of project whichever is later
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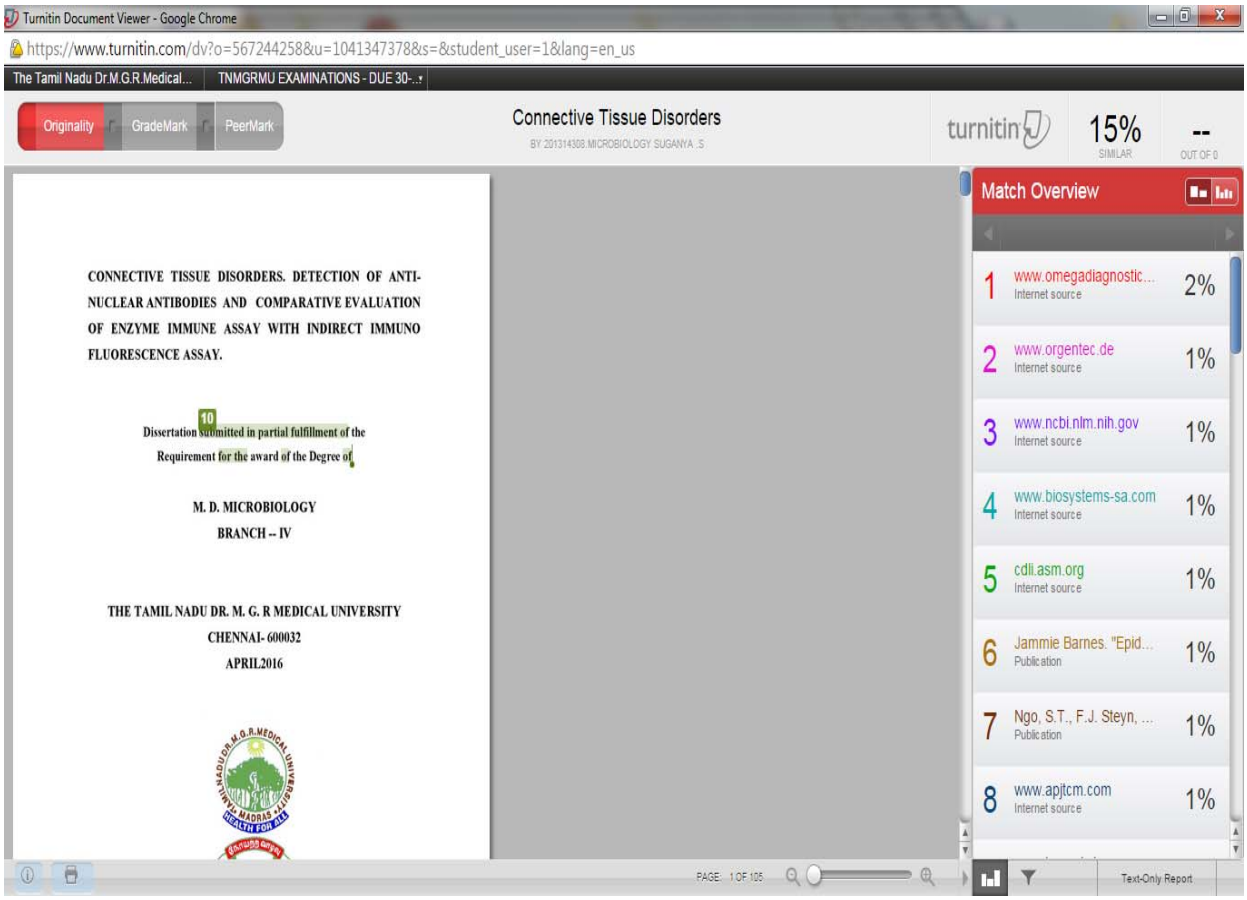
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## DECLARATION

I, solemnly declare that the dissertation titled **"CONNECTIVE TISSUE DISORDERS. DETECTION OF ANTI-NUCLEAR ANTIBODIES AND COMPARATIVE EVALUATION OF ENZYME IMMUNE ASSAY WITH INDIRECT IMMUNOFLUORESCENCE ASSAY"** is done by me at the Department of Microbiology, Tirunelveli Medical College, Tirunelveli.

I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in India or abroad.

This is submitted to the Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

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ABBREVATIONS

| | | |
|---|---|---|
| ANA | - | Antinuclear antibodies |
| CTD | - | Connective Tissue Disorders |
| SLE | - | Systemic Lupus Erythematous |
| SS | - | Sjogren's Syndrome |
| RA | - | Rheumatoid Arthritis |
| DM/PM | - | Dermatomyositis/ Polymyositis |
| AD | - | Auto immune Disease |
| ENA | - | Extractable Nuclear Antigen |
| CLIF | - | Crithidia Luciliae Immunofluorescence |
| MIA | - | Multiplex Immuno Assay |
| HEp-2cell | - | Human Epithelioma type-2 cell |
| FPBC | - | Filter Paper Blood Clots |
| IIF | - | Indirect Immuno Fluorescence |
| ELISA | - | Enzyme Linked Immuno Sorbent Assay |
| FITC | - | Fluorescein isothiocyanate |
| TMB | - | 3, 3', 5, 5'- tetra methyl benzidine. |

# INTRODUCTION

Connective tissue disorders are a group of systemic auto-immune disorders with protean manifestations. They affect multiple organs and tissues revealing a rich constellation of signs and symptoms. Identifying the disease related symptomatology as well as detection of sensitive or specific anti-bodies in the sera of suspected patients, is a must to justify the presence of the disease.

## 1.1Auto-immunity

The daunting role of the immune system is to safeguard the host from infectious diseases and foreign antigens[1] .The survival of immune system, depends on two different types of immune responses; destructive against non-self antigens and protective effect on self antigens[2].

Self-recognition and self-tolerances are the two essential immune mechanisms that protect the body from self-destruction. The inability to silence the immune system to auto antigens leads to autoimmunity and hence auto immune diseases[3].

The presence of reactive T cells against its own body, auto antibodies and inflammatory markers are the fundamental characteristic features of an autoimmune disease. In an autoimmune disease, individual immune response and the various patterns of antibodies produced are well known to vary between persons, places probably population too [4]. Antibody

assessment is an essential part in the serological diagnosis of autoimmune diseases[5].

**1.2 Auto-immune diseases**

Ian Mackay and Macfarlane Burnet were the pioneers in auto immune diseases. The individual immune system has lost its ability to read and accept its own cells and tissues, resulting in a deviant immunological response by both lymphocytes as well as antibodies[6]. It is just a 'friendly fire' against the body's own tissue.

Auto-antigens evokes two type of immune responses ,either cell type specific or organ specific which pave the way for classification of autoimmune diseases into systemic and organ specific.

The systemic types of autoimmune diseases have a variety of auto antibodies. They show greater specificity for certain diseases and include antibodies such as, anti-dsDNA, anti-Sm, anti-ribosomal P auto antibodies in Systemic Lupus Erythematous(SLE), anti-topoisomerases1(Scl-70) in Scleroderma (Scl),anti-CCP in Rheumatoid arthritis(RA),anti-SS-A/Ro, anti-SS-B/La, in Sjogren's syndrome(SS),anti-U1-RNP,anti-PM-Scl in mixed connective tissue disease and anti-Jo-1 in polymyositis or dermatomyositis.

In the organ or tissue specific autoimmune diseases, the auto antibodies are directed against the particular organ or tissue. Thyroglobulin (TGA) and thyroid peroxidase enzyme (TPO) in thyroiditis, insulin and

glutamic acid decarboxylase antibodies in T1DM and anti-mitochondrial autoantibody in primary biliary cirrhosis are few among them.

### 1.3 Prevalence

#### 1.3.1 Global prevalence

A world-shattering report from the American Autoimmune Related Diseases Association (AARDA), released an article in March 2015, represented that autoimmune diseases collectively affect 5-10 percent of the developed world's population. Hence they are a significant cause of chronic illness and death[7].

Among Americans, struggling with autoimmune diseases (AD), more than are 75% are women. AD is one among the 10 leading causes of mortality of women under the age of 65. It is responsible for more than $100 billion in direct health care costs annually.

As the incidence and prevalence statistics for AD are disapproving and not real, there arises a need to perk up the collection and consolidation of these two data. In the USA, the average incidence of SLE ranges from 1.8 to 7.6 cases per 100,000 person-years and the prevalence of SLE ranges from 15 to 50 per 100 000 persons.

The incidence of SLE in USA is similar to those incidences reported throughout the world.

The prevalence of Primary Sjogren's range from 0.1% to 4.6%. Secondary Sjogren's syndrome prevalence as associated with Rheumatoid Arthritis 17.1%, SLE 8-20%, Systemic Sclerosis 14%.

The prevalence of RA is around 1% of world population. For Systemic sclerosis (Scleroderma), incidence rates in the USA is 260 new cases per million; UK 13-48 per million and Australia 86 per million.

The incidence scenario of polymyositis–dermatomyositis ranges from 2 to 10 new cases per million persons in different populations.

Overlap syndromes are considered when a collection of important signs and symptoms of more than one systemic auto immune disease prevailed in the same patient. They are often defined by a particular serological test like extractable nuclear auto antibodies assay.

Sharp, first represented Mixed Connective Tissue Disease (MCTD) as a peculiar type of overlap syndrome. A controversy regarding MCTD as a separate entity still persists. So correct data about its prevalence are not available.

Hence the average prevalence of AD is 4.5%. Out of these 2.7% of males and 6.4% of females were affected. For specific diseases, prevalence ranges from 1%. For all auto immune diseases, the most common mean age-of-onset is 40–50 years[8].

Auto immune disease has emerged as one of the common non-communicable disease.

### 1.3.2 Indian prevalence

The prevalence of SLE in Indian population is 3.2 per 100,000, the overall annual incidence being 5-100 per population. Among them more than 90% of the affected are females.

In India the prevalence of scleroderma is 120 per million. The prevalence of RA in India is 0.75% of the population. Primary Sjogren's among Indian population is an extremely rare entity.

## 1.4 Connective tissue diseases

"Connective Tissue Disease" (CTD) is defined as a spectrum of disorders, which may be either hereditary or acquired; characterized by alteration in structure or deviation in function of one or more of the elements of connective tissue. The elements may be collagen, elastin, or the mucopolysaccharides.

### 1.4.1 Predisposing factors

**A) Genetic Factors**

- HLA genes

  Autoimmune diseases have a strong tendency to be associated with genetic factors, particular HLA specificities. There is a relative risk in individuals harboring certain HLA haplotypes than others who do not have. HLA-DR2, HLA-DR3 confers a modest increased risk in SLE.HLA –DRB1 increase the

risk of rheumatoid arthritis.HLA-DQA1 is highly associated with Sjogren's disease [9].

- Non HLA genes

The protein tyrosine phosphatase gene (PTPN22), expressed in lymphocytes is associated with rheumatoid arthritis, SLE, probably by failing to delete auto reactive T cells in thymus and by ineffective clearance of pathogens

**B) Environmental Factor**-environmental factors that predispose to autoimmunity include

1. Infections by micro organisms (mycoplasma, Epstein Barr virus, rubella virus, in case of RA)

2. Vitamin D deficiency-It has the ability to modify the immune response generated by lymphocytes[10]. The T and B lymphocytes reaction to an immunological reaction is reduced[11,12,13]. The interaction between Vitamin D and human leukocyte antigen molecule leads to epigenetic modifications. Increased sun exposure leads to increased vitamin D synthesis; thereby reducing the possibility of acquiring autoimmunity is seen in SS[14], RA[15] and SLE[16].

3. Exposure to chemicals (silica, trichloroethylene, benzene in case of Sjogrens), xenobiotics or toxins[17]

4. Stress.

**C) Hormones and sex -** The role of male and female sex hormones in autoimmunity includes

- Estrogen: It has a different effect in cell mediated and antibody mediated diseases.

  At physiological concentration it increases the expansion of $CD4^+$ CD25 cells, to tolerate the reactivity of auto antigens and prevents auto immune diseases.

  At the same time, the accelerated maturation of the B lymphocytes and the enormous production of type 2 cytokines from Th lymphocytes by estrogens favor antibody production. This may lead to auto immune diseases.

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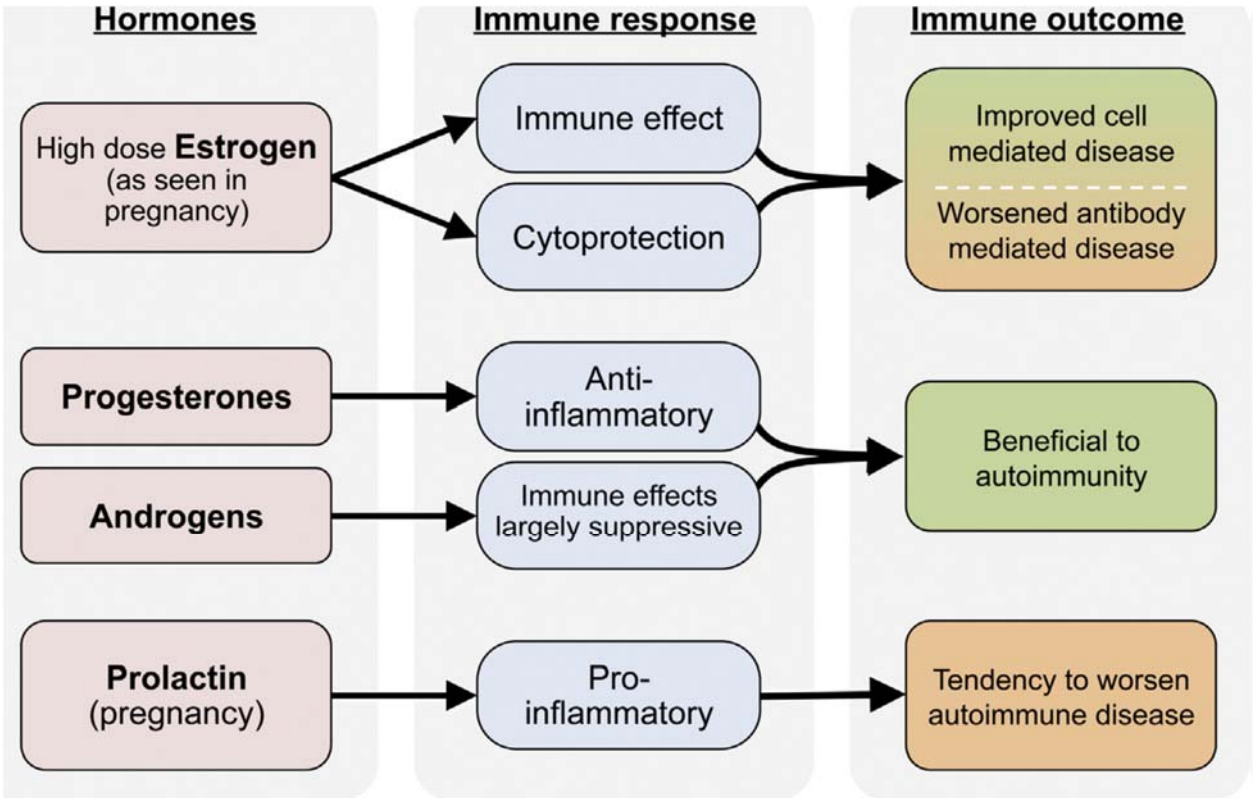


- Progesterone and Androgens: Protective role of both progesterone and androgens in ameliorating autoimmunity are well documented. Progesterone produce markers that are anti – inflammatory, and androgens by reducing the effects of immune reactions.
- Prolactin, synthesized during pregnancy, has the potential to induce early inflammatory markers. This leads to inflammatory changes and later autoimmunity

**D) Other Factors**

**1. Complement deficiencies-** SLE is more prevalent in C4, C1q, C1r, or C2 complement deficiencies as the circulating immune complex (CIC) are not cleared.

**2. Immune system-**In animal experiments conducted; males have immune system that is supppressd[18]. Females show increased immune reactivity[19] and hence are immune competent. This results in greater buoyancy to all types of pathological disturbances, paving way for high immune response. Ultimately women are more vulnerable in developing autoimmune disease[20].

### 1.4.2 Immunopathogenesis

- Molecular mimicry[21]

It is a process in which particular infectious microbes after infecting the individual pave way for the development of auto- immune

diseases. This is because the antigens present in the microbes are quite similar to some host self- antigens. Obviously the response produced by the T and B cell against the microorganism also result in damage to host cells with same molecular structure.

- Loss of suppression

Various types of suppressor cells are present in our immune system to maintain the peripheral tolerance. These cells decrease in number as age advances. The result is that the auto reactive lymphocytes become active leading to autoimmune diseases particularly SLE.

- Epitope spread[22]

The human body is in a state of 'homeostasis because of both of the positive and negative feedback mechanisms. This negative feedback mechanism fails in its role, as the self antigen responsible for the auto immune reactions cannot be eliminated without complete pathological damage in the tissues.

In addition, the destruction exposes the previous hidden antigen aggravating further antibody production. This process is known as epitope spread and it can be either intramolecular spread or intermolecular spread[23]. The auto antibodies to non-protein self antigens such as DNA and phospholipids explains the intermolecular spread

❖ Sequestrated antigens

For various reasons all self –molecules are not expressed; some are sequestered. When they are exposed, the immune system views them as foreign antigen and attacks. Their sites are collectively called as 'immunologically privileged sites'.

❖ Cryptic epitopes[24]

Some of the molecules with three dimensional configuration shelter epitopes within it and prevent from contact from immune system. They are known as 'cryptic epitopes'. When these hidden epitopes are exposed, they are available for immune recognition and binding by antibodies.

### 1.4.3 Pathology of autoimmune diseases

In SLE, auto-antibodies are developed against nuclear components, nucleic acid antigens and cytoplasm. As a result of these auto antibodies, the levels of circulating immune complex (CIC) are elevated. CIC are very small and deposit in the small blood vessels. Therefore immune complex deposition and subsequent complement activation leads to tissue destruction.

Skin lesions in SLE are characterized by following changes[25]: thickening of stratum corneum, separation of basal epidermal layer by hydrolytic enzymes, acute inflammation of blood vessels along with

lymphocytic deposition and islet-like infiltration, fluid accumulation in connective tissues, vasodilatation and extravasations of red blood cells[26].

In kidney, the histopathological changes range from minimal immune deposits in the sub endothelium, sub epithelium to either segmental or global proliferation. In the extreme stage sclerosis is noted[27].

SLE has a varied outcome in pregnancy. The auto-antibodies in SLE belong to IgG and therefore cross the placenta and produce neonatal lupus. Within few weeks the maternal antibodies are eliminated from the blood and hence the disease disappears. Anti-Ro antibodies are associated with inflammation and fibrosis of conducting system resulting in congenital complete heart block.

In Sjogrens disease, cell mediated auto immune response appears to play a main role. Lymphocytic deposition along the ducts and blood vessels seen in almost all salivary, lacrimal glands are usually earliest histopathological findings in this disease. There is an also lymphocytic infiltration of respiratory gastro intestinal and vaginal tract. Plasma cells and B cells are also present[28].

In Systemic Sclerosis (Scleroderma), there is a characterized thickening of skin due to accumulation of connective tissue. The symptoms result from sequential fibrotic changes in tissues and blockade of small capillaries and venules. These are the result of uncontrolled synthesis and accumulation of type 1,3 collagens [29].

In RA, micro vascular injury is observed along with hyperplasia of synovial cells, perivascular infiltration by mononuclear cells. Activated synovial fibroblasts produce enzymes such as collagenases, cathepsins, etc. which degrade the components of articular matrix. Cytokines secreted as a result of the ongoing inflammatory process leads to cartilage and bone damage in addition to systemic manifestations[30].

In polymyositis, the humoral auto immune mediated response attack the muscle capillaries and small arterioles. Immune complexes are deposited in the endomysial capillaries resulting in tissue damage. Perivascular atrophy is followed by necrosis and degenerative fibers throughout the muscle.

### 1.4.4 Clinical features and criteria's

#### 1.4.4.1Systemic lupus erythematous

It is a chronic disease involving multiple organ system: features including rash dermatitis, arthralgia, small vessel vasculitis, vague abdominal pain, nephritis, scleritis, neuropathies pericarditis, myocarditis, pulmonary hypertension,etc [31],. The hallmark is the production of auto-antibodies against a number of self antigens such as DNA, histones, ribonucleoprotein, erythrocytes and platelets etc. SLE is the most prevalent immune complex disease.

##### 1.4.4.1.1 Diagnosis of SLE

Around 11 criteria have been defined by the AMERICAN COLLEGE OF RHEUMATOLOGY. If 4 out of 11 is present[32,33] ,the diagnosis of SLE is

made with 98% specificity and 97% sensitivity. The criteria includes 1) butterfly rash on malar prominence 2) discoid rash 3) sensitivity eruptions to sunlight 4) ulcers in the oral cavity 5) inflammation of the joints 6) inflammation of serous membrane 7) symptoms and signs of renal pathology as well as 8) nervous system 9) disorders involving erythroid & myeloid lineage 10) abnormal immune reactions and its consequences 11) anti-nuclear antibodies

**1.4.4.2 Sjogren's disease.**

Henrik Sjogren, a Swedish ophthalmologist first described keratoconjuctivitis with swelling of major salivary glands[34].Sjogren syndrome is sub divided into primary and secondary. Manifestations include photophobia, xerostomia, atrophic rhinitis, xerotrachea, parotitis, Raynauds phenomenon, hyper pigmentation, arthralgia, bronchitis, bronchiolitis, peripheral neuropathy, anemia etc.

The revised International criteria proposed by American-European Consensus Group (AECG) 2002[35], was updated in 2012by Shiboski et al[36] includes detection of specific auto antibodies, salivary gland biopsy, SICCA with ocular staining >3.

**1.4.4.3. Scleroderma**

The criteria were developed by the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR). Extensive skin thickening of the fingers extending proximal to the

metacarpophalangeal joints is sufficient for a patient to be classified as having scleroderma. If this is not present, minimal skin thickening of fingers, finger tip lesions telangiectasia, abnormal nail fold capillaries, pulmonary hypertension, interstitial necrosis, Raynauds phenomenon and presence of scleroderma related antibodies are all considered[37].

#### 1.4.4.4. Rheumatoid arthritis

American College of Rheumatology and European League Against Rheumatism (2010) proposed a classification criteria[38]. Other presentations include joint tendon destruction, rheumatoid nodules, pleural effusions, interstitial fibrosis, nerve entrapment etc.

#### 1.4.4.5. Dermatomyositis and polymyositis

Heliotrope skin rash on upper eye lid, flat red rash seen in trunks, scaly eruptive Gottrons papules on knuckles, paronychial erythema and nail fold bleeding, muscle pain and tenderness[39].

### 1.4.5 Protean presentation- Drive for diagnostic methods

As varied symptoms and signs are present in CTD, diagnosis based only on clinical presentation is intricate. It should be emphasized that auto immune diseases cannot be diagnosed by a single test, rather by a group of investigations. This includes direct immunological test like IIF, ELISA, Flow cytometry etc, or indirect immunological associated like CRP, ESR, ferritin etc

or basic profiles such as complete hematological, coagulation, muscle enzyme and so on.

Not only these tests support in the diagnosis and management of sufferers of AD, they also play a role in assessing prognosis and to indicate the extremity of pathological changes in the organs.

### 1.4.5.1 ANA- Its milestones.

Klemperer, Pollack and Baehr worked together and acknowledged that Connective tissue disorder includes systemic lupus erythematous as one of its entity in 1941[40].Various researches had been done on SLE patients. This led to the observation of an entirely different cell structure in marrow specimen of a SLE patient in 1948 by Hargraves and his colleague. This previously unknown cell was referred as LE cell.

These are polymorphonuclear leukocytes that have a wonderful capacity to phagocyte the bare nuclei of other leukocytes with the help of auto antibodies[41]. It thus provides a way to opsonize the liberated nuclear material of the target cell. This identification fronted subsequent research for ANA. Consequently ANA was categorized into two broad types.

### 1.4.5.2. ANA –Two broad subtypes

#### 1.4.5.2.1 Auto antibodies to DNA and histones

This includes antibodies directed against the single and double stranded DNA (dsDNA) discovered in 1957 and the anti-histone antibodies discovered in 1971, to be the markers for SLE and drug induced SLE respectively[42,43,44].

#### 1.4.5.2.2. Auto antibodies to extractable nuclear antigens (ENA)

These auto antibodies target nuclear antigens other than DNA and histones. They include auto antibody to Smith antigen[45] (the first ENA detected), RNP, SSA/Ro, SSB/La, Scl-70, Jo-1and PM-1. Originally there are extracted from nuclei with saline and hence the name[46,47].

### 1.4.5.3 ANA detection

Detection of auto antibodies to nuclear antigens is important as its presence in the sera is taken as one of the parameters to confirm the CTD in a patient. Also the presence of ANA suggests to subtype the auto antibodies, to confirm specific CTD[48].

#### A] Indirect Immunofluorescence

Still now immunofluorescence remains to be a basic technique in the assay of auto-antibodies. The auto antibodies get adhered to the substrate HEp-2, producing fluorescence images of different pattern. Detection of ANA is sometimes misinterpreted and there is also possibility cross-reactions.

3%-13% of the individuals carry ANA without having disease manifestations. Their presence in a screening assay will give a high prevalence rate. In addition to these pitfalls the level of ANA in an autoimmune patient is relatively high during active phase but seldom detected when he is on remission.

Each and every fluorescent picture is peculiar. They represent a particular type of CTD.

**Normal titer ranges for antinuclear antibodies is age - dependent**

- $1^{st}$ and $2^{nd}$ decade of life: less than 1 in 20
- $2^{nd}$ to $6^{th}$ decade: less than 1 in 40
- $7^{th}$ decade and older: less than 1 in 80

Reporting should include pattern of fluorescence along with titer value.

- In homogenous staining, nucleus is evenly stained. This indicates antibodies to histones and deoxyribonucleoproteins.
- In speckled pattern , fine to coarse staining of nuclear material occurs, indicating Smith antigen, ribonucleoproteins etc.,
- In peripheral staining pattern, nuclear lamina or pores stained. This points the presence double stranded (ds) DNA, rheumatoid factor, and anti-phospholipid.
- In nucleolar staining, the nucleoli appear as large stained particles within the nucleus, Scleroderma and Sjogren's are associated with this pattern.

- In centromere, fluorescent pattern, numerous scattered particles are seen indicating systemic sclerosis variant known as CREST syndrome.

The positivity rate of anti-nuclear antibodies in SLE is 95-100%: in Scleroderma; a 65-90%, in Sjogren's 50-60%; in rheumatoid arthritis 25-30%. The positive result needs to be confirmed by other specific diagnostic methods.

**B] ELISA**

This procedure has acceptable specificity and sensitivity. This assay which decreases the time needed to screen quite large number of samples needs to be considered. Performing this technique is very simple. It can also be programmed but needs large number of samples from the normal person as well as from auto immune persons to confirm.

This enzyme immune assay may be either general assay which detects antibodies of wide-ranging specificity similar to immunofluorescence or antigen explicit assay that detects ANA and reacts with a single auto antigen. The later includes the ENAs like dsDNA, SS-A/Ro, SS-B/La, Scl-70,etc.

Almost all rheumatological classification criteria include the detection of auto antibodies. After screening by enzyme immunoassays and immunofluorescence, the positive results needs more specific test to confirm the clinical diagnosis.

### 1.4.5.4 Methods to arrive specific ANA

The methods used spot out specific –ANA includes: Cirthidia luciliae Immunofluorescence (CLIF), Farr assay (a radio-labeled assay), Counter current immune electrophoresis (CIE), Passive haemagglutination assay (PHA),Western blot ,Dot blot, Line blot Immunoassay, Multiple Immunoassay(MIA), Flow cytometry, Antigen microarray.

**CLIF assay**

CLIF assay is an immunofluorescence test, done by using trypanosomes with intracellular kinetoplast, containing dsDNA in high concentration. When correlated with ELISA, CLIF sounds good in terms of specificity, in SLE detection. CLIF is easy to perform and also there is no problem of meddling with antibodies to single-stranded DNA. Its sensitivity is also high.

**Farr radioactive assay**

This is a radio-labeled procedure which measures the antibodies through precipitation assays. It is specific but extracts a lot of time. It demands experienced persons to run the test and involves the use of radioactive material. It is not used in most laboratories.

**Double immune diffusion**

This detects more than one antibody at a projected time; its cost factor is acceptable and detection of true negatives by this double diffusion method is high. This method detects true positives at a lower rate.

**Counter immune electrophoresis**

Similar to double immunodiffusion, in detecting multiple antibodies simultaneously. The high specificity and cost – effective are also equal to the above procedure.

**Passive haemagglutination**

The assay identifies all the true positives but pose a low screening value in identifying true negatives. It fails to delineate patients having anti-Sm from anti-U1-RNP antibodies. It is a semi quantitative- test.

**Western blot**

More sensitive than double immunodiffusion and counter immune diffusion, but low in specificity. Studies conducted on apparently healthy individuals frequently showed positive results.

**Line immunoassay**

It is another qualitative test which is easy to perform and can be done in a short period of less processing time. Sensitivity and specificity of Line immunoassay and ELISA are nearly the same. Programmed interpretation makes it easier.

**Multiple Immunoassays**

MIA allows the screening of many samples for specific ANA in a given time. This assay is well-organized in identifying the specific ANA and easy to carry out than routine immunofluorescence screening. In addition, the

rate of false positivity is very low, removes subjective variation and is more capable than usual ELISA.

**Flow cytometry**

This gives quantitative results and is cost-effective. A fully automated procedure giving high true positives, the major problem is only a single test can be done at a point.

**Microarray**

A nano-technology technique for simultaneous measurement of multiple ANA. It can be used to search and observe novel auto antibodies. It's an automated procedure with high sensitivity and specificity. These methods are not commercially popularized.

### 1.4.6 Treatment

The goals of the treatment are to:

- Alleviate the symptoms
- Shorten the ongoing autoimmune damage
- Improve the body's ability to overcome the pathological effect[49].

Treatment modality is based on the presenting disease, clinical signs and symptoms

Some auto immune patients may need increase in hormones like thyroxine, insulin or vitamins like $B_{12}$ that are deficit in their body. Autoimmune disorders affect the hematopoietic cell lineage leading to auto

immune hemolytic anaemia and other hematological features. They may depend on repeated blood transfusions.

The immune system's response to auto antibodies are checked by a group of immunosuppressive medicines. Such medicines may include corticosteroids (such as prednisone) and nonsteroid drugs such as azathioprine, cyclophosphamide, mycophenolate, sirolimus, or tacrolimus

In refractory cases, IV Immunoglobulin and plasma exchange done.

Certain novel drugs like belimumab, ocrelizumab,epratuzumab and abatacept are showing promising results and future research are needed[50].

### 1.4.7 Prevention

Autoimmune disease is skyrocketing, but conventional treatment has little to offer.

Primary prevention requires the

- Identification of vulnerable persons;
- Updating the immunopathogenesis of disease; and
- Framing safe and logical interventions.

Screening for identification of individuals at risk for autoimmune diseases include

• Identification at genetic level, including MHC focus and non HLA genes.

• Rationalization of assays for auto antibodies and T cells;

•Development of reliable screening assays;

• Shared facilities for large-scale screening efforts

Some preventive aspects also consider the identification of environmental triggers.

Autoimmunity Centers of Excellence**,** Stem Cell Transplantation for Autoimmune diseases are conducting clinical trials to ensure safety and to evaluate effectiveness of the treatment regimens for scleroderma, and SLE.

**MALAR RASH IN SLE**

**ERYTHEMATOUS LESIONS IN BOTH PALMS**

**GOTTRONS PAPULES ON KNUCKLES**

## AIMS AND OBJECTIVES

1) To detect anti-nuclear antibodies in suspected Connective Tissue Disorder (CTD) patients by Indirect Immunofluorescence (IIF) assay & generic Enzyme Linked Immuno Sorbent Assay (ELISA).

2) To determine the type of CTD by observing the type of fluorescent pattern.

3) To compare the two techniques–generic assay with the Gold Standard IIF

# REVIEW OF LITERATURE

Autoimmune diseases essentially comprise a range of diseases in which the immune response to self-antigens results in damage or dysfunction of tissues. Criteria including the identification of a target antigen, the presence of antibodies and/or T cells in the target organ, and the transfer of disease to animals by cells or antibodies allow a disease to be classified as autoimmune[51, 52].

The immune system includes multiple cell types from a single lineage of lymphoid progenitor. These play different roles in acquired as well as innate immunity. Both these immunity interact in response to auto antigen and aggravate autoimmune responses.

## 3.1 Burden of auto-immune diseases

### 3.1.1 Global scenario

Reported prevalence frequencies range from 20-240 per 100,000 persons, and reported incidence ranges from 1-10 per 100,000 person years[53].

The female to male ratio differs with aspect to age in SLE. The female-to-male ratio in adults is 10-15:1, in older –onset SLE it is 3:1 , and in children it is 8:1 Because of better detection of early stages of disease, the incidence of SLE has nearly tripled in the last 40 year[54].

A community based survey was conducted in Birmingham (United Kingdom). It reported a prevalence of diagnosed SLE in women ages 18 to 65

years of 54 per 100,000, and new cases are added upon during each screening, increasing the data to 200 per 100,000[55]

In a study done by DE Furst et al the SLE incidence rate (2003–2008) was 7.22 cases per 100,000 person-years. The annual prevalence of SLE (per 100,000 individuals) varied from 81.07 in 2003 to 102.94 in 2008[56].

Primary Sjogren's syndrome prevalence rate range from 0.1% to 4.6%. The incidence and prevalence rates are significantly higher in women than men, approximately 20:1 with a peak incidence in the fifth and sixth decades of life.

Secondary Sjogren's syndrome prevalence as associated with rheumatoid arthritis is 17.1%, SLE is 8-20% and systemic sclerosis is 14 %.

A prevalence study was observed among the population in Turkey by Kabasakal Y and noted the old and new cases of primary Systemic sclerosis in women from 2$^{nd}$ decade to 7$^{th}$ decade as 1.56 per 100,000 [57]. The annual incidence was found to be 3.9 per 100,000 populations for primary SS, in a study by Pillemer, SR, Matteson et al.

Monaco et al., conducted a retrospective review of Italian-origin patients excluding pediatric and adolescent populations. He classified the patients on the basis of diagnostic criteria for Scleroderma and found the prevalence ratio as female to male ratio of 9.7:1[59]. Increased predominance is noted in females

In Taiwan, Kuo et al., the first reported population study for systemic sclerosis in Japan, estimated the annual incidence rate was 10.9 cases per million and the prevalence were 56.3 cases per million[60].

As per 2010 study the world wide prevalence of RA was 0.24% (95% CI 0.23% to 0.25%), DALYs increased from 3.3 million (M) (95% CI 2.6 M to 4.1 M) in 1990 to 4.8 M (95% CI 3.7 M to 6.1 M) in 2010, explains the severity of the disease. This increase may also be attributed to population growth and increase in aging.

The world prevalence of RA might be around 0.3–1.2%[61].

Mordvinov GV et al, found RF more often occurs in healthy women than in men, particularly at the age of 17-39 years.

RA affects approximately 1.3 million in the US[62].

**3.1.2 Indian scenario**

Malaviya AN conducted a study in the north Indian population reported a prevalence of SLE ranging from 14 to 60 per 100,000[63].

As per another study in India, the prevalence of SLE is lower at 3.2 per 1,00,000[64]. Minz *et al.*, reported a prevalence of Systemic sclerosis of 120 cases per million[65] among adults and children in northern India. Debashish Dandaet al, the reported prevalence varying between 0.5 per cent and 2 per cent among various populations in India.

Primary Sjogren's syndrome (SS) is rarely reported from India[66]

**3.2 Gender variation**:

Ninety percent of patients are women affected by autoimmune diseases belong to child-bearing age.

Due to hormonal effect, the prevalence of SLE is far more in females than in males, especially after puberty. The female to male ratio is 10-15:1 in adults, 3:1 in older onset SLE, 8:1 in children. Additionally, gender may produce different characteristics in the manifestation of SLE[67,68,69].

The incidence and prevalence are 20:1, higher in women than men in Sjogren's.

In Systemic sclerosis also, the female preponderance is seen, the ratio being 5-14:1. In RA, women are three times more affected than men.

Polymyositis and Dermatomyositis the ratio is 2.5:1 representing female being affected more

**3.3 Racial variation**

The prevalence of SLE is higher among people in Asia, as well as in Afro-Americans, Afro-Caribbean's and Hispanic Americans. Low prevalence is seen with Americans migrated from European countries. Estimated incidence rates vary from 1 to 25 per 100,000 in American continents, Europe and Asia.

A study was designed by Malgorzata E. Krzyszczak et al, between African-American and Caucasian Systemic Sclerosis patients. They proved ANA patterns were entirely diverse between these two societies. Incidence rates and prevalence estimates are in close range for Europe, the United States,

Australia, and Argentina .Their estimated prevalence is 150–300 cases per million. But a considerable lower prevalence is documented in Scandinavia, Japan, the UK, Taiwan, and India.

Rheumatoid arthritis also shows racial variation. American Indian tribes and Alaskan Indians succumb to this auto immune disease more commonly than those living in African and Asian countries[70,71].

**3.4 Urban rural variation**

The auto-immune disease appears to be more common in urban than rural areas[72].

A prevalence study was carried out in a rural population near Delhi in India. It revealed a point prevalence of 3 per 100,000. This is a much lower figure than reported from the rural population in the western countries. The values were 12.5 per100, 000 adults in England; 3 to 39 per 100,000 in Finland; 4 and 124 per 100,000 in USA[73].

Rheumatoid arthritis is present in all group of population except the rural Africa[74].

**3.5 Childhood prevalence**

Childhood SLE is also a multisystem connective tissue disorder. This occurs in children below 16 years of age. It is the second commonest among the pediatric rheumatic disorder. Juvenile Idiopathic Arthritis ranks first followed by childhood SLE. Common age of presentation is from 5-16 years

(mean age – 12 years). Like adult SLE, female predominance (5:1) is also noted here.

### 3.6 SLE and hypothyroidism

Pyne and Isenberg analysed the association of hypothyroidism in SLE patient. They reported a higher prevalence of hypothyroidism in patients with SLE (5.7%) than in the normal population (1%) [75].

Miller and co-workers in their study had noted a significantly higher association (6.6%) of hypothyroidism in patients with confirmed SLE [76].

Rudrajit Paul, of Kolkotta, conducted a study on known SLE patient and evaluated the presence of anti-TPO antibody. He found 11 out of 16 (68.7%) patients with SLE had anti‑TPO antibody positive, which was statistically significant ($P<0.05$). This result sounds similar to the study found in Japan[77].

### 3.7 Familial predisposition

Auto-immune diseases may be inherited; studies are there to substantiate this. The genes responsible for the pathogenesis of this systemic rheumatic diseases can be transmitted vertically. Previously autosomal dominant inheritance was suspected but the interaction of common genes plays a role[78].

At times autoimmune diseases run in families[79]. In a study conducted in Taiwan it was found that 2nd and 3rd degree relatives with Lupus were diagnosed to suffer than 1st degree individuals.

### 3.8 Prevalence among pregnant women

As connective tissue disorders show a rise in reproductive period, pregnant mothers are not exceptional. Microchimerism occurs during pregnancy between mother and fetus and vice-versa. There are of two types' fetal microchimerism and maternal microchimerism. This has been considered to be a possible stimulus for autoimmune disorders.

Rosenburg[81] evaluated first, second and third trimester serum samples in addition to umbilical cord sample after each delivery of 100 healthy pregnant women. 76 non-pregnant women were the controls and fluorescent pattern produced on HEp-2 cells at titers of greater than or equal to1:80 are compared. Among these samples, the first, second third trimester gestation and cord sera were found to be 18, 21, 21 and 15, positive for ANA respectively.

In a Italian study when the serum dilution titer was kept as 1:20 for immunofluorescence assay, the antibodies did not differ in the control (52 non pregnant) and the study group (84 pregnant).

The prevalence of auto-antibodies to nuclear antigens was compared between women with normal pregnancy, with those who had previous three abortions in a study by Garcia et al[96], found to be 6.6% in normal and 30% in patients with miscarriages.

In a study done on patients with recurrent spontaneous abortions, the association of anti nuclear antibodies was found to be 13.6% in comparison with control population which showed 1.2% by Bahar et al[80].

This test was in close correlation with the study done by Eroglu. Among 72 patients with repeated miscarriages 9 of them had low levels of ANA arriving a percentage of 13.2%.

But in a particular study between Argentine women with first trimester fetal loss and women with no bad obstetric history, there was no significant diference[82].

In a study conducted in India, comparison was made between patients with single abortion, recurrent abortions and fertile controls.ANA detection by both ELISA and HEp-2 immunofluorescence concluded that recurrent aborters had increased frequency (35%) of anti cardiolipin antibodies as against single aborters (12.5) %.

**3.9 ANA detection by immunofluorescence**

Ghosh et al[40], performed a ANA screening dilution for SLE by a commercial kit for IIF assay. 213 serum samples were screened. They were collected from 94 healthy controls, 43 Systemic Lupus Erythematous patients, 37 Rheumatoid Arthritis patients and 39 patients suffering from Dermatomyositis / Polymyositis. At a dilution of 1:80, it was found that, 95.3 per cent of the SLE patients and 4.3 per cent of healthy individuals were positive for ANA. This ultimately indicated that 1:80 dilution titer of immunofluorescence was the best screening dilution to distinguish SLE patients from healthy individuals.

Tan et al[83] have shown that 1:160 was the best dilution which distinguished SLE patients from healthy individuals.

In a study conducted among sufferers of systemic sclerosis. 217 individuals tested and 58 (27%) positive for antibody for nuclear antigen. Among the fluorescent image patterns obtained speckled nuclear (42%), followed by nucleolar (13%), homogenous nuclear (2%) and centromere pattern.

Susan s Copple et al study represented the necessity for automated procedure and validated it. The recommendation of the ACR to use IIF as the preferred method has triggered the development and validation of automated systems for ANA determination[84].

Automated pattern interpretation of HEp-2 ANA was first described by Perner et al[85]. NOVA View is a computerized digital reflection analyzer system. It was designed to simplify the interpretation of ANA fluorescent patterns on the substrate HEp-2 cells. This is based on measured Light Intensity Units (LIU) and pattern recognition.

Clinically defined serum samples from patients suffering from SLE (n= 50), rheumatoid arthritis (n = 44), Scleroderma (n= 35), Sjogren's syndrome (n =19), and polymyositis ( n= 10) were included. In addition, 99 healthy adult donor sera which consisted of 70% female and 30%male between the ages of 19 to 59 years of age were tested

All samples were processed manually. The results were read by both the Nikon manual microscope and NOVA View, archived images with software version 1.0.2 by a board certified medical technologist. Screening was done at dilutions of 1: 40 and1: 80 and the results NOVA view and manual IIF were compared. The NOVA view agreement with manual fluorescent images interpretation at 1:40 dilution were 94.8% positive agreement; 97.4% negative agreement; 96.5% total agreement. At 1:80 dilutions their positive, negative & total agreements were 92.9%, 97.4% & 96.2% respectively. Spearman's correlation was excellent.

Sebastian et al viewed homogenous nuclear pattern in 45.5%, speckled nuclear pattern (35.6%) among ANA positive persons. but Sunitha et al observed a different percentage of pattern in fluorescent assay using HEp-2 cell and cytoplasmic granular being the most reported

**3.10 ANA detection by ELISA**

Binder et al[32], done an assay to improve the utility of immunoassay-based ANA screening. He combined a multiplex immunoassay with a *k* nearest neighbor (kNN) algorithm for computer-assisted pattern recognition. Sera from patients with various rheumatic diseases and non-diseased patients were screened. Results were positive for 94% of the systemic lupus erythematous (SLE) patients by both HEp-2 cell based enzyme linked immunosorbent assay (ELISA) and multiplex immunoassay. The kNN

algorithm declared the pattern for 84% of the already diagnosed antibody-positive SLE patients.

For controls the multiplex method found fewer positive results than the ELISA screen. None of the disease pattern was proposed by the kNN algorithm for most of controls. He concluded by mentioning the automated algorithm could identify SLE patterns earlier itself**.**

**3.11 Comparison of ANA detection by IFAT and ELISA**

A comparative study by Divate et al, from 96 systemic rheumatic disorder patients, Enzyme Linked Immunosorbent assay showed positivity in 53 samples and among them only 10 showed positivity for immunofluorescence. The sensitivity and specificity arrived were 90.7% and 85.7%. The positive and negative predictive values follow as 89.1% and 87.8%.

In another comparative evaluation between ELISA and IIF, the sensitivity of SLE (74%), Systemic sclerosis (72%), primary Sjogren's syndrome (89%), mixed connective disorder (100%) and inflammatory myopathies (39%) were noted

Priyadarshini[98] et al done a study to compare ELISA with IIF. 125 patient samples from clinically diagnosed auto immune disorders, 25 samples of healthy persons for negative control and 25 samples of proved ANA positive auto immune diseased patient for positive control are assembled.

IIF and ELISA assays showed sensitivity of 98.3% &71.43% respectively. The specificity for IIF found to be 93% and for ELISA it was 86.84%. IIF showed more positives than ELISA.

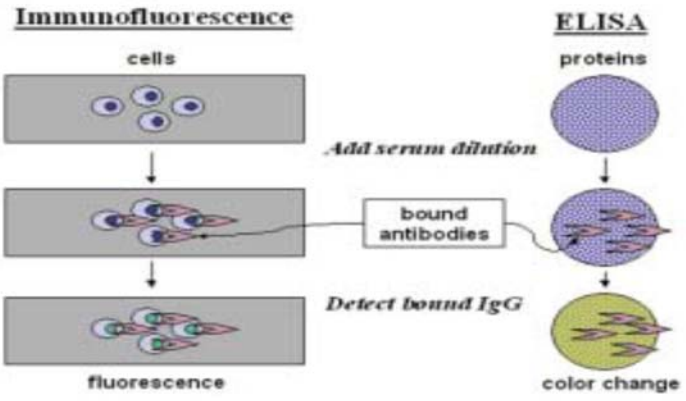
Gneiwek et al, reported that ANA ELISA documentation of the sensitivity and specificity are nearly equal and in fact specificity is to some extent more. Out of the screened 86 sera ,74 were positive by both tests ANA IIF& ANA ELISA .4 of the sera tested positive for ANA ELISA were negative with IIF[86].

In a rheumatologic assay conducted by Dipti et al[87] of Bangladesh, there were 27 (67.5%) ANA IFA positivity among 40 cases and 12 (27.5%) ANA ELISA positive. The differences were highly significant (p value< 0.001). ANA by IIF was positive in 100% SLE. Similar percentage result was also found by El-Chennawai.

Fawcett et al[88] showed presence of anti nuclear antibodies in 157 patients by IIF and only 95 patients by ELISA.

**3.12 Specific ANA detection techniques**

C.luciliae kinetoplasts in indirect immunofluorescence had been considered as the best substrate for the detection of anti-ds DNA in a study done by Ballou et al; when the positive cut-off was increased from 20 to 43%, he noticed a100% correlation between C. luciliae and ss DNA binding in SLE sera[89,90]. In a study designed by Sommerfield, Crithidia luciliae assay was similar to radio labeled Farr assay.




**Indirect immunoenzyme method**

From the filter paper blood clots (FPBC) Chopra A detected anti-nuclear antibodies using indirect immunoenzyme method, considering IIE as less expensive since only light microscopy is needed.

In his pilot study of 21 known cases of systemic auto immune rheumatic disease, sera and FPBC were compared by IIE and IIF. IIE showed hundred percent sensitivity for lupus. Analyzing the Kappa statistic of agreement, it came as 1 (perfect) and 0.4 (fair) for FPBC-IIF and FPBC-IIE respectively when compared with IIF. Homogenous, speckled and centromere were the fluorescent images obtained. Among the 10 paired samples tested 8 shows similar results between IIF and FPBC –IIE.

**Passive haemagglutination**

Sasaki T et al conducted a study to detect the anti-DNA antibodies by indirect haemagglutination and hemolysis test using the chromium chloride-treated sheep erythrocytes. He suggested that the antibodies to DNA correlates

with the presence of disease severity in patients with systemic lupus erythematous (SLE).

Niemhom analyzed the diagnostic efficacy of passive haemagglutination and immunofluorescence. Among 169 sera of suspected SLE 59 sera were positive and 91 sera were negative by both methods. By haemagglutination assay 5 were found negative but positive by immunofluorescence. Low haemagglutination titers were negative by immunofluorescence in his evaluation.

The correlation between the results obtained by both methods were highly significance with contingency coefficient of 0.61 and correlation coefficient between the results of 78 sera positive by both or either method was 0.74 (p value less than 0.001).

**Multiplex Immunoassay**

Hayashi et al, compared Multiple Immunoassay with immuno fluorescence and found the sensitivity and specificity of the IIF method at titer 1:40 were 92% & 65% respectively. But for MIA at a cut-off of 0.6, the sensitivity 93% and specificity 79% were obtained. Both showed similar results at high titer and higher cut –off[92].

**Flow cytometry**

Flow cytometry assay uses auto antigen coated fluorescent beads, also known as Reflex ANA has salient points like simultaneous detection of several antigens, automation, cost effectiveness and high sensitivity. Shovman

et al. conducted a screening on connective tissue disorders and showed the sensitivity of fluorescent beads testing for 113patients with SLE was about 80% when correlated ELISA ANA which came around 82.3%. In another study conducted by Rouquette FB showed an excellent concordance value of 99.1%-100% with enzyme linked immmunosorbent assay.

The disadvantages of fluorescent beads assay were also analyzed. The proteins covalently binding to the solid phase produce conformational changes may lead to elimination of native epitopes. The difference in the size and charge of the fluorescent beads alters the binding of auto antibodies. With reference to a study done by Waterboer, human sera display false poly reactivity to multiple antigens in the FB assay.

**Line immunoassay**

In a prospective and retrospective double blind study undertaken on the South Indian population who were referred for detection of ANA, the ANA was tested by indirect immunofluorescence and Line immune assay methods. Of the 319 samples evaluated, 101 (31.7%) showed positive in both ANA-IIF &Line immune assay; 21(17.2%) positive for ANA –IIF but negative for Line immunoassay. Within the ANA-IIF negative seen in 197 samples, 31 (15.7%) found positive in Line immunoassay.

This study closely resembles the one done by Sulcebe of Albania and somewhat closer to study by Slater. Since definite correlation was

appreciated between the ANA patterns and the specific group of antibodies, screening can be done by assessing the IIF-ANA patterns.

Damoiseaux et al. performed a comparative trial study of line immunoassay with counter immuno electrophoresis and enzyme immune assay for detecting ENA. LIE had a high sensitivity and specificity than CIE and EIA. The true positive rate for line immune assay, enzyme immunoassay and CIE were 17.9%, 11.4% and 8.3%. He concluded that line immunoassay may be considered a good alternative in screening specific auto antibodies.

Ghirardello A did a diagnostic performance to validate the usefulness of anti nuclear antibody testing in myositis. He utilized a commercial line blot assay and found, Line blot could be an appropriate serological test in the diagnostic workup for myositis. This is similar to the observations made by Rönnelid J[91]

**Antigen Microarray**

The nano-technology based technique using pre-synthesized antigens provides complete automation. It may be find a fruitful place for the discovery and evaluation of novel antibodies[93].

**3.13 Treatment of Auto immune diseases**

The current approach in AD can be broadly categorized into two categories

- treating the symptoms
- halting or suppressing the immune response

Hydroxychloroquine reduces the exacerbations and increases the life expectancy of SLE patient, making it the keystone of SLE management[94].

Nonsteroidal anti-inflammatory drugs (NSAIDS) or low-potency immunosuppression medications beyond hydroxychloroquine and/or short courses of corticosteroids are also used to treat SLE.

Persons with autoimmune diseases who are prone to thrombosis can be treated with acetyl salicylic acid. Low dose heparin is also used, but aspirin has the capacity to cross placenta and of use in gravid women. In autoantibody positive, treatment with both prednisolone and aspirin increases the success rate more than 80%.

Intravenous immunoglobulin (IV Ig) can be safely used if patients have reported side effects to aspirin &heparin. Moreover it also protects the fetus from the cytotoxic maternal immune response by neutralizing the effect.

Lymphocyte immune therapy also used in gravid women with bad obstetric history as it causes suppression of natural killer cell activity.

Vitamin D3, an immunomodulatory agent is thought to reduce the production of T-helper cytokines

The monoclonal antibody belimumab is a B-lymphocyte stimulator–specific inhibitor. It reduces the antibody production and thereby reduces the disease severity.

Rituximab treatment leads to B cell depletion. This finds a special place in treatment of rheumatoid arthritis

At present, there are no curative agents for Sjogren's syndrome. The treatment of this disorder is essentially symptomatic.

Cyclophosphamide, mycophenolate sodium and similar agents are used for treating grave manifestations of Sjogren's syndrome.

Researchers are going on in assessing the role of anti-CD22, anti-BAFF anti-T cells etc.

### 3.14 Hematopoietic-cell transplantation

The normal life expectancy is usually near normal for most of the autoimmune disease sufferers. But some patients suffer relentless, therapy unresponsive, unchecked autoimmune damaged. Hematopoietic-cell transplantation (HCT), a potential therapy is the ultimate choice for those people. HCT involves the administration of hematopoietic stem cells (HSC), which can self-renew and allow all lineage cells to produce cells.

These can get rid of self-reactive memory B cells and T cells. Thence the immune system reverts to near normal status. HCT might also allow regeneration of tissues that are destroyed by autoimmune disease.

## MATERIALS AND METHODS

The present study was conducted at the Department of Microbiology, Tirunelveli Medical College, Tirunelveli from February 2015 to June 2015.

### 4.1 STUDY GROUP

A total of 90 serum samples were collected from clinically suspected connective tissue disorder cases in adults

#### 4.1.1 Inclusion criteria

1) All suspected cases of CTD attending TVMCH.

#### 4.1.2 Exclusion criteria

1) Pediatric population(age less than 12 years)

2) Patients seropositive for HIV, HBsAg.

#### 4.1.3 Ethical clearance

Ethical clearance was obtained from the college ethical committee before the commencement of the study.

#### 4.1.4 Informed Consent

Informed consent in their understandable language was obtained from reliable informants of patients who participated in the study.

#### 4.1.5 Proforma

The proforma was filled with the details like name, age, sex, ward, clinical diagnosis and other parameters significant to the present study.

Symptoms related to the corresponding organ system involvement in CTD were recorded in the questionnaire.

### 4.1.6 Sample

A total of 90 non-duplicate serum samples were collected from the study group. Around 3 ml of blood sample was collected from suspected cases of connective tissue disorder. Serum was separated and collected in serum vial. The specimen was properly labeled with serial number, name of the patient and date of collection.

### 4.1.7 Sample storage

Sample were immediately stored for IIF &ELISA at -80 $^{o}$ C.

## METHODS

## 4.2 INDIRECT IMMUNOFLUORESCENCE

The assay of anti-nuclear antibodies by IIF was done using BIOSYSTEMS KIT (ANTI-NUCLEAR ANTIBODIES HEp-2).

### 4.2.1 Principle of the method

Anti-nuclear antibodies (ANA) present in the serum of the patient will get attached to the analogous antigens present in the given substrate HEp-2 cells. Immunological reaction occurs between both and antigen –antibody complexes are formed. They are then allowed to combine with a fluorescein labeled anti-human immunoglobulin. The resultant fluorescent pattern is read by using a fluorescence microscope.

### 4.2.2 Composition

A] SLIDES: each well is coated with HEp-2 cells

B] POSPHATE BUFFER SALINE (PBS), (10X):sodium phosphate 112.5 mmol/L,potassium phosphate 30 mmol/L, sodium chloride 1.15mol/L, sodium azide 0.95g/L, Ph 7.2 .

C+] ANA –Hep-2 POSITIVE CONTROL: Human serum containing anti-nuclear antibodies (ANA) homogenous nuclear pattern, sodium azide 0.95 g/L.

C-] NEGATIVE CONTROL: Healthy human serum, sodium azide 0.95g/L.

D] IgG FITC/EVANS: Goat anti-human IgG conjugated with fluorescein isothiocyanate (FITC), Evans blue 0.01g/L, sodium azide 0.95g/L.

E] MOUNTING MEDIUM: Mowiol 12%, Glycerol 30%, Tris 20 mmol/L, sodium azide 0.95g/L.

F] BLOTTING PAPER

{Human sera used in the positive and negative controls found to be negative for the presence anti-HIV and anti-HCV antibodies, as well as for Hepatitis B surface antigen}.

**4.2.3 Storage:**

Reagents are stored at 2 – 8 degree Celsius.

**4.2.4 Reagent preparation:**

PBS: The reagent PBS is diluted 1/10 with distilled water

**4.2.5 Additional equipment needed:**

1] For incubation a moist chamber,

2] Tray for washing purpose,

3] Cover slips 24x60 mm,

4] Fluorescence microscope equipped with a 495 nm excitation filter and a 525 nm emission filter for FITC image perception

**4.2.6 Procedure:**

1) The reagents and sample were brought to room temperature.

2) Samples were diluted 1/80 in PBS.

3) One drop [25microlitre] of diluted sample or control was placed on each slide well.

4) The slides were incubated at room temperature [15-30$^0$C] for 30 minutes into a moist chamber.

5) Sample drops were drained off by gently tapping the inclined slide. Cross contamination of the sera was avoided.

6) The slide was gently rinsed with diluted PBS.

7) The slide was thoroughly washed by immersing in a washing tray filled with PBS for 5 minutes. PBS was changed and again washed.

8) The slides were dried off carefully by using the blotting paper provided. The substrate was kept moist along the procedure.

9) One drop of reagent D was placed on each well. The slide was incubated at room temperature [15-30$^0$C] for 30 minutes into a moist chamber.

10) Washing [step 7] and drying [step 8] were followed.

11) Drops of reagent E was placed along the slide and covered with the cover slip. Care was taken to avoid forming bubbles.

### 4.2.7 Reading

The slides were examined using the fluorescence microscope (250-400X), immediately. The specific fluorescent staining was observed and the pattern described at recommended dilution was considered as positive.

The patterns may be nuclear including homogenous, peripheral, speckled, nucleolar, cetromere, or cytoplasmic. When none of the above specific staining was observed, the result was considered negative for these auto antibodies.

### 4.2.8 Quality control

Positive control C+ and Negative control C- were tested along with the patient samples, in order to verify assay performance.

Positive control gave the described specific staining.

Negative control did not give any specific staining.

### 4.2.9 Assay characteristics

The IgG FITC/EVANS conjugate was calibrated versus the International Standard of the WHO for sheep anti-human immunoglobulin conjugated with FITC.

The specificity of the ANA-HEp-2 positive control had been verified against the AF/CDC1 reference serum from the Centre for Disease Control.

## 4.3 ENZYME LINKED IMMUNO SORBENT ASSAY

ANA detection by ELISA was done using ANA-Ease ELISA KIT produced by GENESIS DIAGNOSTICS. It is a rapid ELISA method to detect

ANA of the IgG immunoglobulin type. The wells are coated with antigens such as dsDNA, histones, SSA/Ro, SSB/La, Sm, Sm/RNP, Scl70, Jo-1 and centromeric antigens. Auto antibodies present in patients sera against these antigens were detected.

**4.3.1 Principle:**

Diluted serum samples are incubated with an aforesaid antigen extracted from HEp-2 nuclei immobilized on micro titer wells. After washing the unbound serum components, rabbit anti-human IgG conjugated to horseradish peroxidase is added to the wells, and this binds to surface bound antibodies in the second incubation.

Unbound conjugate is removed by washing, and a solution containing 3, 3', 5, 5'-tetramethyl benzidine (TMB) and enzyme substrate is added to trace specific antibody binding. Addition of stop solution terminates the reaction and provides the appropriate Ph for color development. Optical densities of the standard controls and samples are measured using a micro plate reader at 450 nm.

**4.3.2 Materials included in the kit:**

**1]** Micro plate: 96 wells in 12x8 break apart strips, all wells were pre-coated with an antigen mixture containing dsDNA, histones, SSA/Ro, SSB/La, Sm, SM/RNP, Jo-1,centromere and other antigens extracted from HEp-2 nuclei.

2]Reagent 1: sample diluents, 10mM Tris –buffered saline, Ph 7.2 with antimicrobial agent, 50ml, (blue), and ready to use.

3] Reagent 2: wash buffer 100Mm Tris buffered saline with detergent, pH 7.2, 100ml, concentrate (X10)

4] Reagent 3: conjugate rabbit anti-human IgG conjugated to horse raddish peroxidase in protein stabilizing solution and anti-microbial agent, 12ml, ready to use

5] Reagent4: TMB substrate aqueous solution of TMB and hydrogen peroxide, 12ml, ready to use

6] Reagent 5: stop solution 0.25M sulphuric acid, 12ml, ready to use

7] Standards: 2ml of 10 mM Tris-buffered saline containing human serum IgG antibodies to nuclear antigens, ready to use

8] Positive control: 2ml of 10Mm Tris-buffered saline containing human serum antibodies to nuclear antigens, ready to use

9] Negative control: 2ml of 10mM Tris-buffered saline containing normal human serum, ready to use

**4.3.3 Other equipments needed**

Test tubes for dilution

Graduated cylinder for preparing wash buffer

Precision pipettes

EIA automated washer

Distilled or deionized water

EIA micro plate reader with 450nm and optional 620nm

### 4.3.4 Storage

The kit was stored at 2-8$^0$C.

### 4.3.5 Preparation of reagents:

Wash buffer was diluted 1:9 in distilled water to make sufficient buffer for the assay run.

### 4.3.6 Assay procedure:

1] Patient samples were diluted in 1:50 in sample diluents (10microl serum plus 0.5ml diluents)

2] 10 U/ml standard, the negative and positive controls and the diluted patient samples were dispensed into appropriate wells

3] Incubated for 20 minutes at room temperature.

4] After 20 minutes, the well contents were decanted and the wells were washed 3 times with automated washer. Wells were not allowed to dry out.

5] 100microl of conjugate was dispensed into each well. Incubated the wells for 20 minutes at room temperature.

6] After 20 minutes, the well contents were discarded and the wells were washed 4 times with wash buffer. Care was taken, not to allow wells to become dry out.

7] 100microl of TMB substrate was dispensed into each well. Incubated the plates for 10 minutes.

8]100 microl of stop solution was dispensed into each well. The stop solution was added into the wells in the same order as the TMB substrate.

9] The optical density (OD) of each well at 450 nm was read in a micro plate reader within 10 minutes.

**4.3.8 Qualitative determination**

Results were negative when the sample OD ≤10 U /ml standard

Results were positive when the sample OD> 10 U/ml standard

**4.3.9 Performance characteristics**

Anti nuclear antibody immunoreactivity was evaluated using ANA Ease and immunofluorescence at 1:80 dilution. Performance yielded the data as sensitivity 90%, specificity 93%, positive predictive value 90%, negative predictive value 93%, and accuracy 91%.

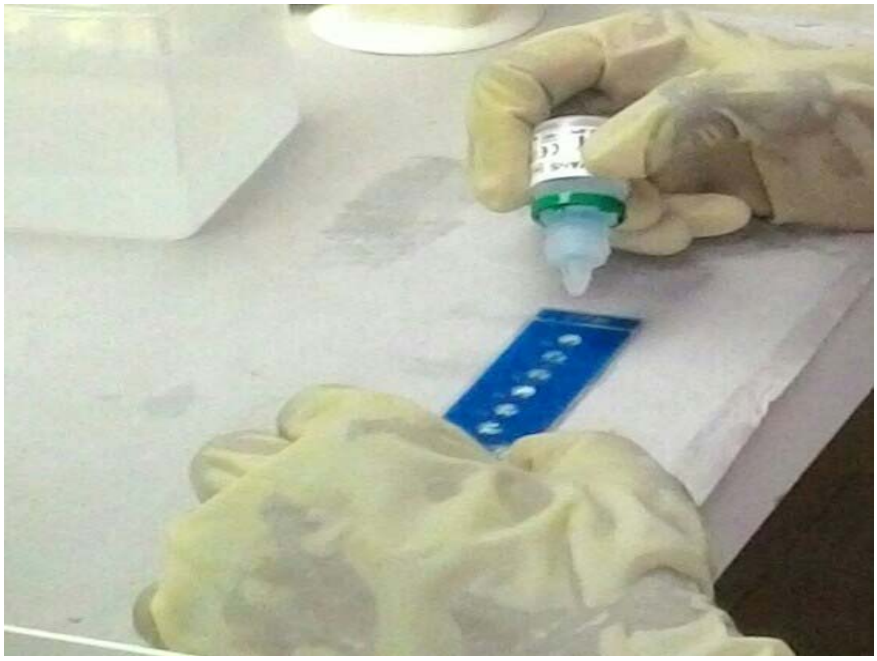
**4.3.10 Quality control**

- Absorbance of positive control should be higher than 1.496
- Absorbance of negative control should be lower than 0.087
- Cut-off standard (10U/ml) should be higher than 0.953.

## FLUORESCENT MICROSCOPE

## IIF ASSAY REAGENTS

**HEp-2 SLIDE (6 WELLS)**



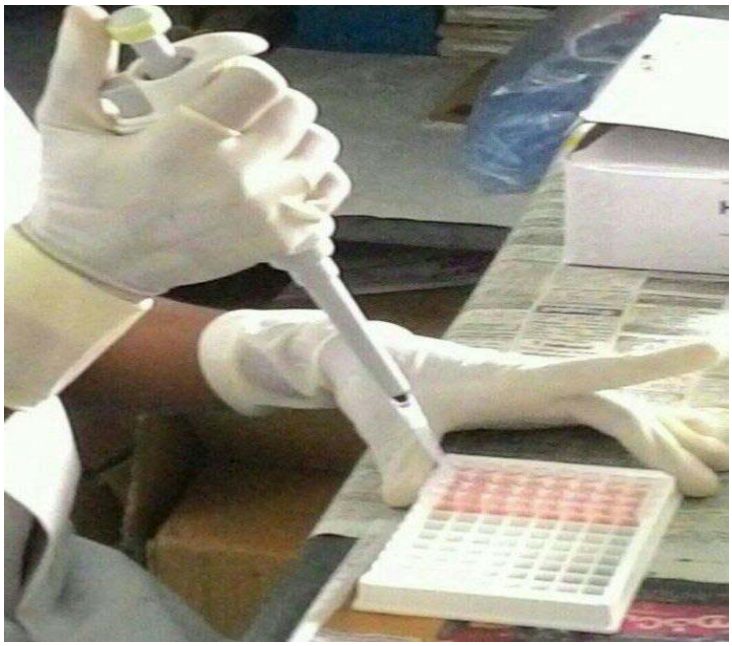
## AUTOMATIC MICRO PLATE ELISA WASHER

## WASHING STEP

**ANA ELISA KIT**

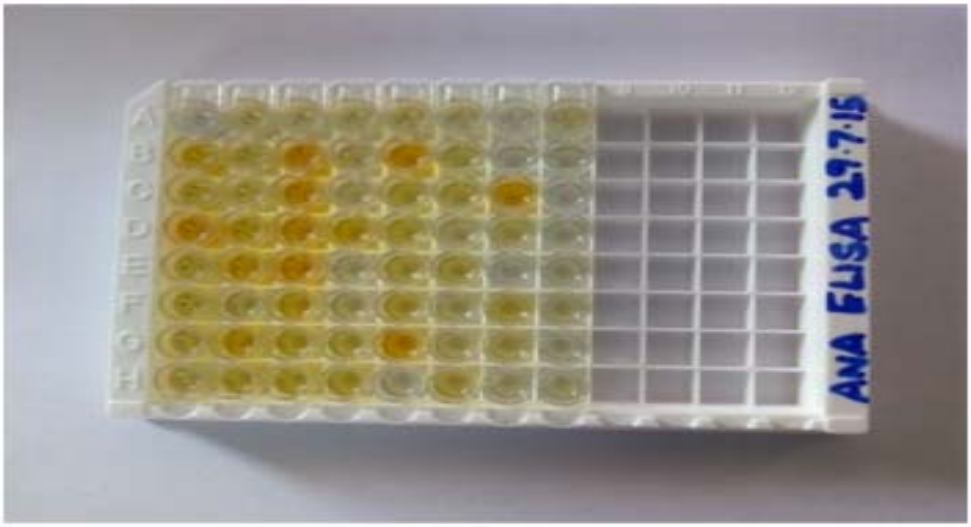

**ELISA WELLS**

**ELISA PROCEDURE**



**ELISA READER**


**ANA- ELISA POSITIVES**



# 5. RESULTS

## 5.1 Statistical Analysis

Data regarding the clinically diagnosed Connective Tissue Disorder patients were analyzed in terms of percentages. The sensitivity, specificity, positive predictive value, negative predictive value of ELISA were evaluated. Assessment were made by applying SPSS statistical software. The difference were considered to be statistically significant when the p value obtained was less than 0.05.

## RESULT ANALYSIS

**Table-1**

**Distribution of samples based on clinical diagnosis.**

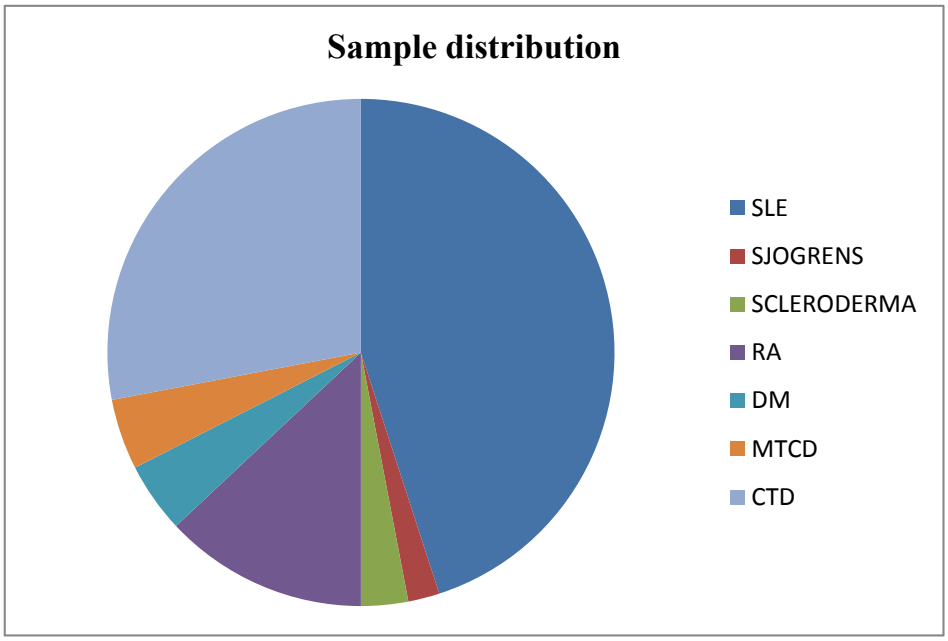
| Clinical Diagnosis | Samples | % |
|---|---|---|
| **Systemic Lupus Erythematous** | 40 | 45 |
| **Sjogrens** | 2 | 2 |
| **Scleroderma** | 3 | 3 |
| **Rheumatoid arthritis** | 12 | 13 |
| **Dermatomyositis** | 4 | 4.5 |
| **Mixed Connective Tissue Disorder** | 4 | 4.5 |
| **Suspected Connective Tissue Disorder** | 25 | 28 |
| **Total** | 90 | 100% |

In this study of 90 suspected Connective tissue disorder patients,40 samples were suspected to be Systemic Lupus Erythematous. Around 25 patients were provisionally diagnosed to suffer from systemic autoimmune etiology,but difficult to specify.They were assigned as suspected connective tissue disorder.

12 patients were suspected to suffer from Rheumatoid arthritis.Dermatomyositis and Mixed connective tisssue disorder were diagnosed in4 samples each, followed by 3in Scleroderma and 2 in Sjogren's.

**Figure-1**

**Distribution of samples based on clinical diagnosis.**




Nearly half of the clinically suspected cases are SLE.(45%).Suspected connective tissue disorder (28%), occupying one-third area.

Rheumatoid arthritis presentation was seen in 13% of the samples.

Dermatomyositis and mixed connective tissue disorder constitute 4.5%, followed by Scleroderma (3%) and Sjogren's (2%).

**Table-2**

**Age and sex wise distribution of the study group**

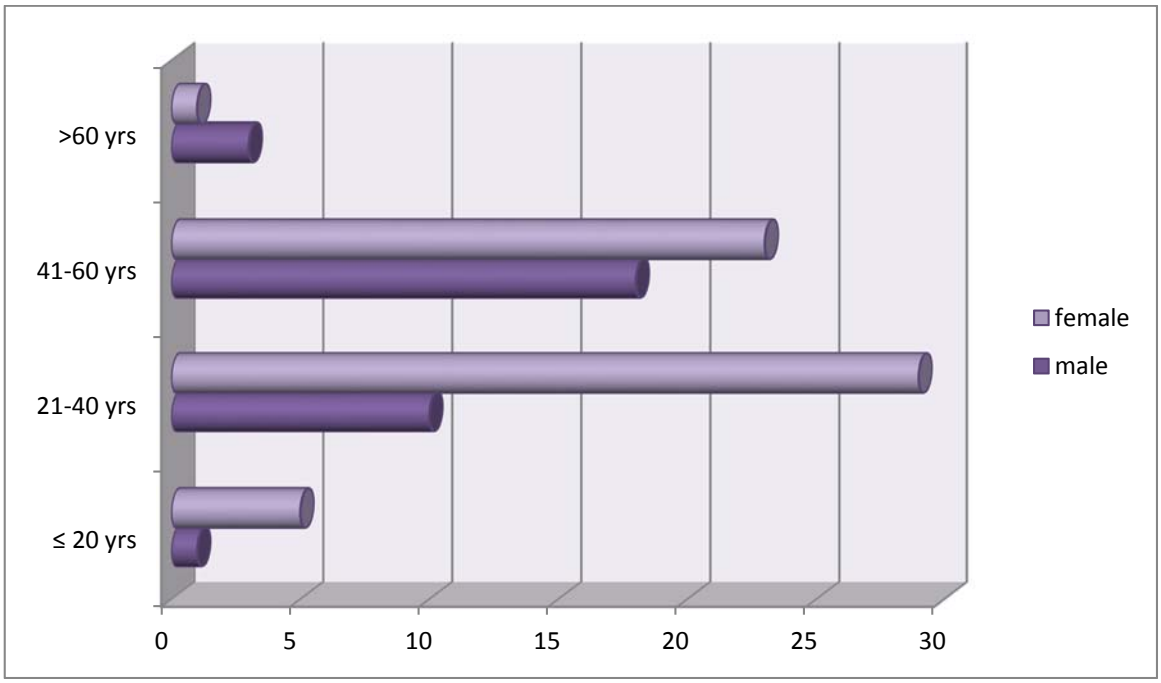
| Age (years) | Male | | Female | | Total | |
|---|---|---|---|---|---|---|
| | No | % | No | % | No | % |
| ≤20 | 1 | 3.2 | 5 | 8.5 | 6 | 6.7 |
| 21-40 | 10 | 31.3 | 29 | 50 | 39 | 43.3 |
| 41-60 | 18 | 56.2 | 23 | 39.5 | 41 | 45.6 |
| >60 | 3 | 9.3 | 1 | 2 | 4 | 4.4 |
| Total | 32 | 100 | 58 | 100 | 90 | 100 |

This table shows the age wise distribution of males and females. Only one male is of 20 years of age. Between the ages 21-40 there are 10 males and from 41-60, there are 18 males. Only 3 samples were present, beyond the age of 60 years. So majority of the suspected males belong to 41-60 years.

Among the females, 5 patients were in the age of 20 years. Majority of the female patients are in between 21-40 years of age. There are about 29 females in the reproductive age. 23 patients are present in between 41-60 years of age. After 60 years only one sample was found.

**Figure-2**

**Age sex wise distribution of study sample**




Most of the clinical samples are between 21-60 yrs. Among males, 31.3% of males were in the age between 21-40 years and 56.2% of samples lie between 41 -60 years. Majority of males were in the age group of 41-60 years.

More prevalence of age among suspected females is from 21-40 years,comprising 50% of the female samples.this is followed by 39.5 % of the females between the age of 41-60 years. Only 8.5 % and 2% of the females suspected to have CTD ,among the test samples were around 20 years and beyond 60 years respectively.

**Table-3**

**Sex wise distribution of different clinical types of CTD**

| Sl. No | Clinical types | Male | % | Female | % |
|---|---|---|---|---|---|
| **1** | **SLE** | 14 | 44 | 26 | 45 |
| **2** | **Sjogrens** | - | - | 2 | 3.5 |
| **3** | **Scleroderma** | 1 | 3.1 | 2 | 3.5 |
| **4** | **R A** | 6 | 19 | 6 | 10 |
| **5** | **Dermatomyositis** | 2 | 6.3 | 2 | 3.5 |
| **6** | **MCTD** | 2 | 6.3 | 2 | 3.5 |
| **7** | **Suspected CTD** | 7 | 22 | 18 | 31 |
| | | 32 | 100 | 58 | 100 |

14 (44%) of the male patients and 26 (45 %) of the female patients were suspected to suffer from SLE. Sjogren's syndrome is diagnosed only in 2 (3.5%) of the female samples and not recorded in males .Scleroderma is noted in 1 male and 2female patient and the prevalence percentage in this study is 3.1% and 3.5% respectively

Regarding rheumatoid arthritis 6 male and 6 female were diagnosed to have the disease. Among the male and female samples the prevalence is 19% and 10%.

2 (6.3%) male patients were suspected to have Dermatomyositis and MTCD each. Among the females 2(3.5%) suffered from Dermatomyositis as well as MTCD.

The undiagnosed CTD accounts to about 7 (22%) in males and 18 (31%) in females.

**Figure-3**

**Sex-wise distribution of clinical samples.**

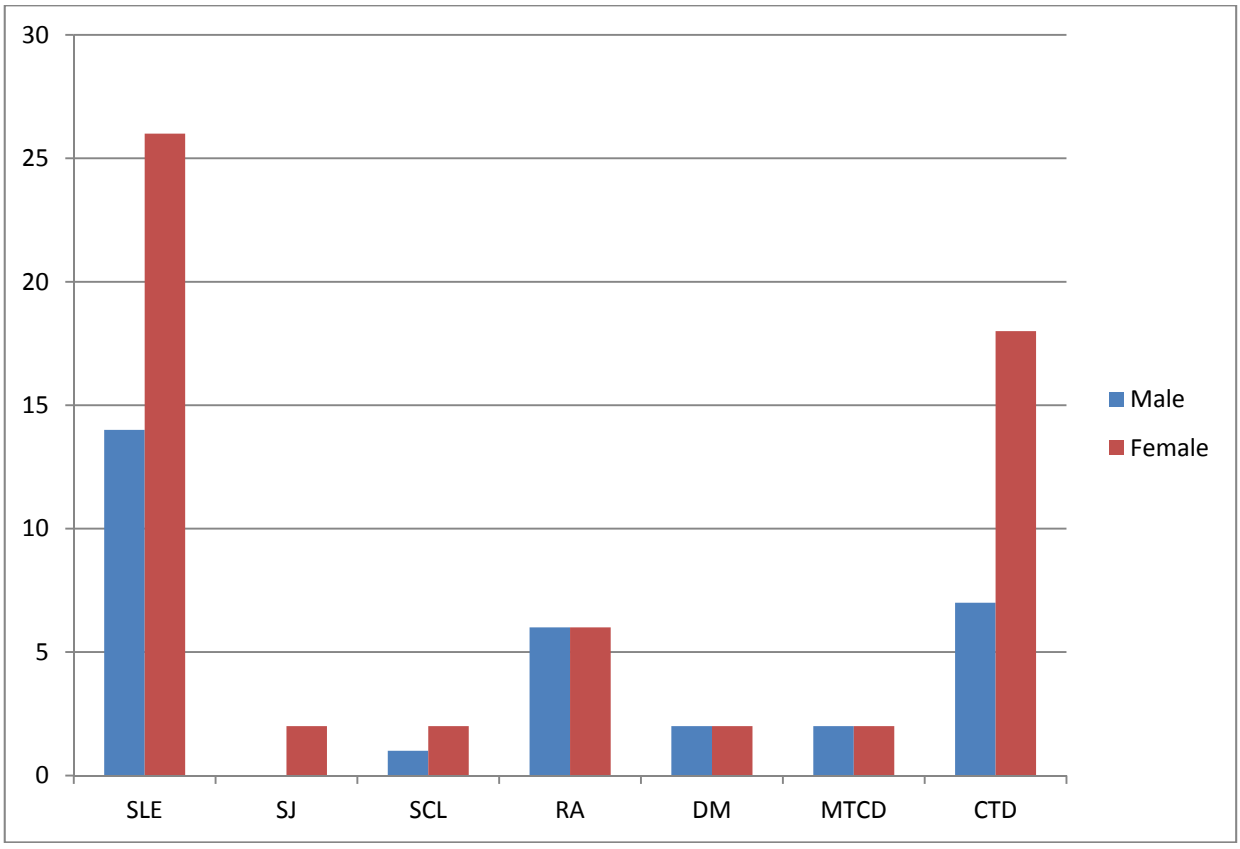


**Table-4**

**Distribution of positive family history among clinical samples**

| | | ANA result | |
|---|---|---|---|
| | | **negative** | **Positive** |
| **Family History** | **negative** | 61 | 25 |
| | **positive** | 0 | 4 |

Out of 90 samples, 4 ANA positives had similar illness among their family members.The Fischer exact p value is less than 0.009, significant.Association exists between family history and prevalence of anti nuclear antibodies.

**Figure -4**

**Presentation of family history among samples**

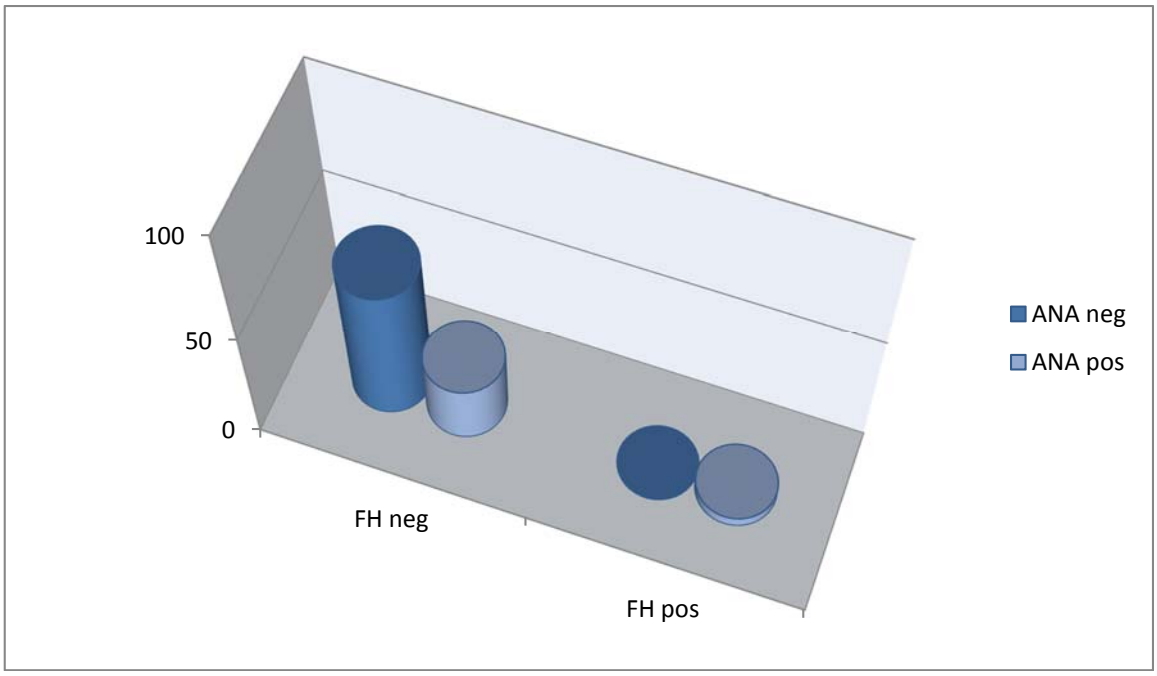


**Table-5**

**Distribution of Bad Obstetric History among females**

| | **ANA positive(20)** | | **ANA negative(38)** | |
|---|---|---|---|---|
| | **No** | **%** | **No** | **%** |
| **BOH positive** | 5 | 25 | 2 | 5.2 |
| **BOH negative** | 15 | 75 | 36 | 94.8 |

( p<o.o42 ) - Significant.

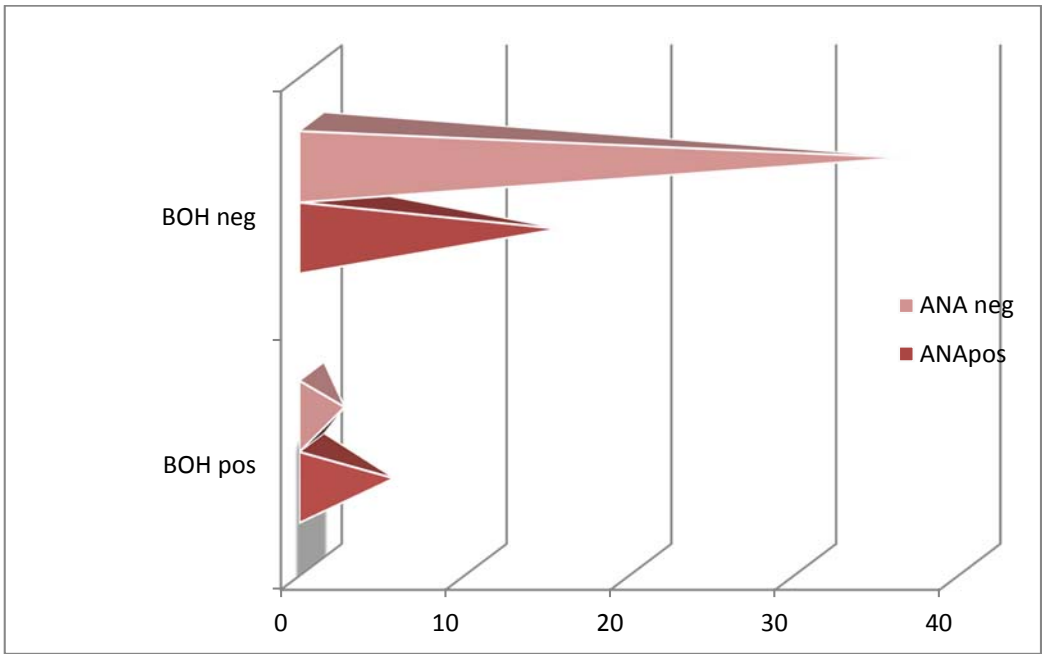
The table shows the number of females having bad obstetric history among seropositive samples.5 cases of BOH were positive for ANA, while 2 BOH were negative for ANA.

15 women, who documented no history of any miscarriages, were found to have auto antibodies.36 females were both negative to ANA and BOH.

The Fisher exact p value is < 0.042.it indicates that the two variables, BOH and ANA have association.

**Figure-5**

**Presentation of BOH among women in CTD**




The BOH positivity rate among ANA positive samples were 25%; the negativity among seropositives were 75%.

Among 38 negative samples, 2 cases were having a history of fetal loss. 36 cases, whom did not have BOH, also did not have antinuclear antibodies.

**Table-6**

**Corelation of positivity with clinical diagnosis**

| Clinical diagnosis | ANA positivity | |
|---|---|---|
| | No | Percentage |
| **Systemic Lupus Erythematous** | 20 | 69% |
| **Sjogren's** | nil | Nil |
| **Scleroderma** | 2 | 6.8% |
| **Rheumatoid Arthritis** | 2 | 6.8% |
| **Dermatomyositis** | 3 | 10.4% |
| **MTCD** | 1 | 3.5% |
| **Suspected CTD** | 1 | 3.5% |
| **Total** | 29 | 100% |

Out of the 29 samples,majority were suspected SLE.

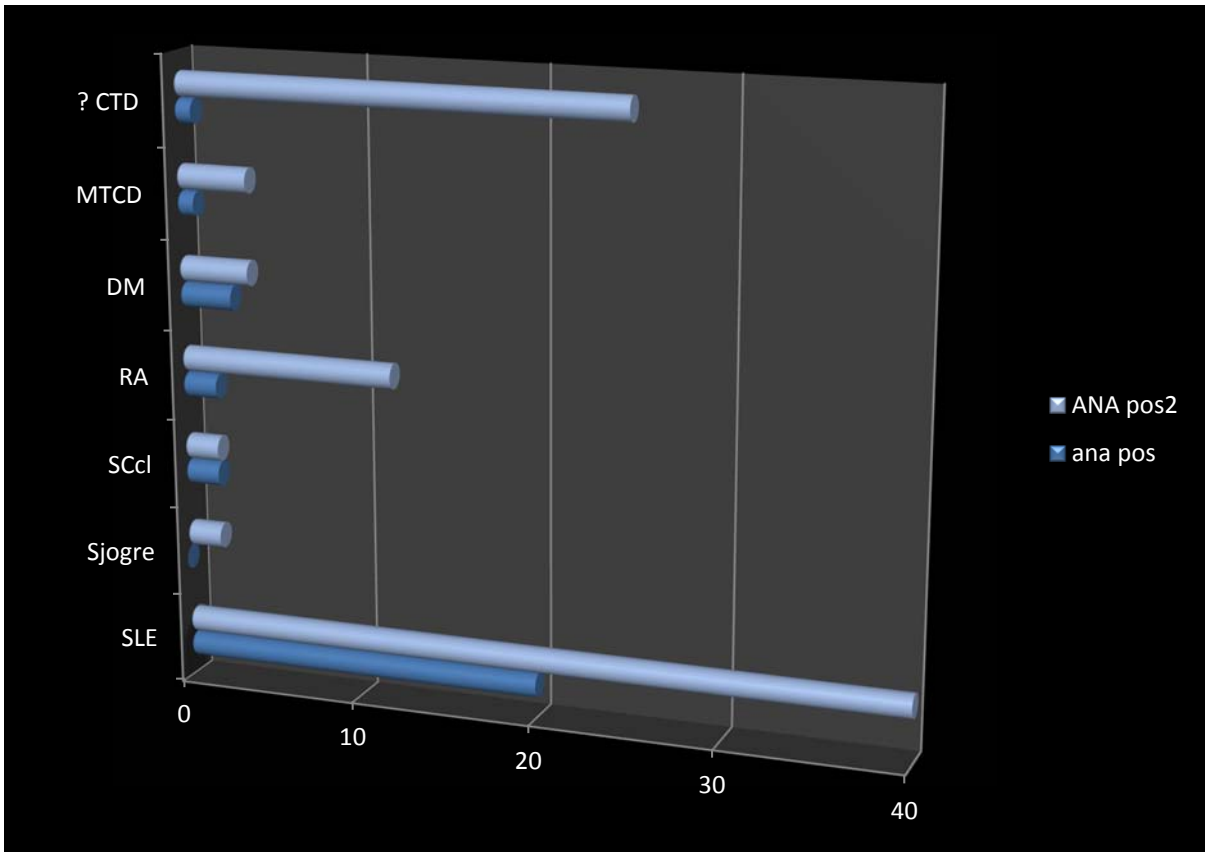
Dermatomyositis were 3; Rheumatoid arthritis and scleroderma were 2 each.

MTCD and suspected CTD were one each.

2 cases suspected to suffer from Sjogren's were found to have negative for the presence of ANA.

**Figure-6**

**Representation of ANA positive samples among clinically diagnosed**




69% of ANA positive samples were suspected SLE cases. Dermatomyositis accounts for 10.4% of seropositives.

6.8 % were clinically diagnosed as Rheumatoid arthritis and Scleroderma each.

Mixed Connective Tissue Disorders and suspected Connective Tissue Disorders constitute 3.5% each.

**Table -7**

**Comparison of clinical manifestations among samples**

| Samples | Skin | Renal | CNS | CVS | JOINT | GIT | RS |
|---|---|---|---|---|---|---|---|
| **Male** | 23 | 9 | 4 | 3 | 30 | 7 | 6 |
| **Female** | 44 | 9 | 9 | 4 | 54 | 24 | 17 |

Among the 90 samples screened, the most common is bone manifestation including arthralgia, early morning stiffness, numbness seen in 30 males and 54females.This is followed by skin manifestations comprising of maculopapular rash, skin thickening in 23 males and 44 females

7 males and 24 females suffered from gastro intestinal manifestations. The features including constipation, diarrhea and heart burn. Respiratory manifestation like cough, dyspnea and wheeze were seen in 6 males and17 females.

9 males and 9 females reported renal symptoms like facial puffiness, haematuria, loin pain & anuria. Central nervous system and cardiovascular system manifestations were noted in minimal patients only.

**Figure -7**

**Distribution of clinical manifestation**

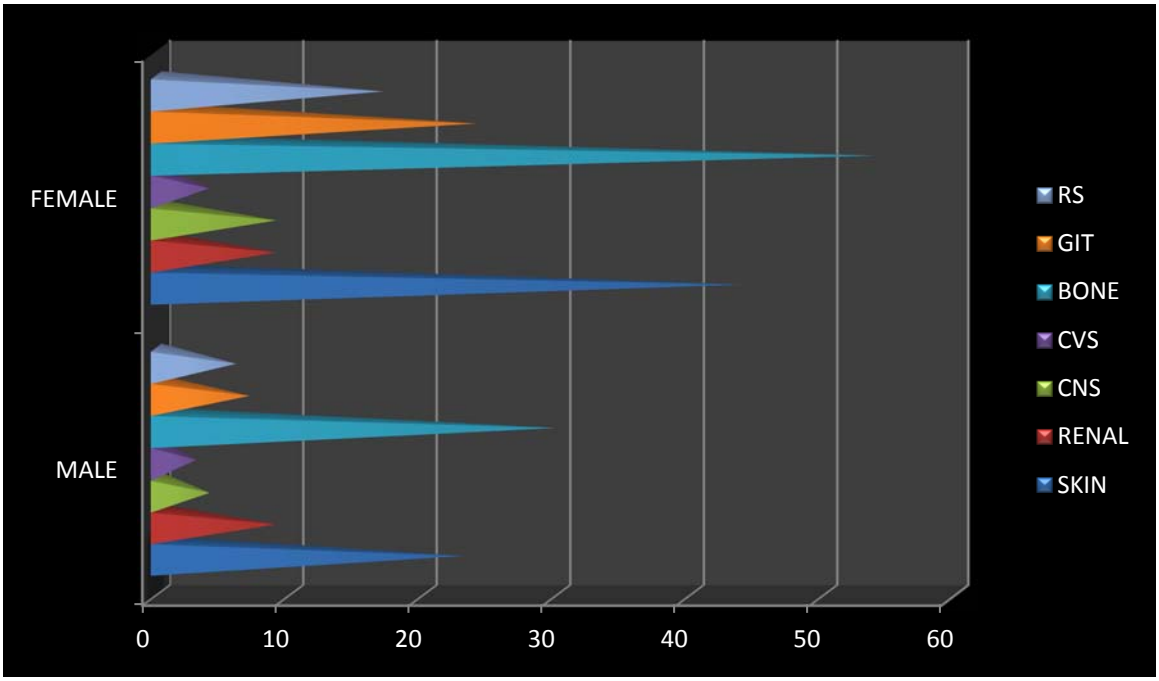



**Table -8**

**Distribution of Clinical manifestations among seropositivity**

| | ANA positive | |
|---|---|---|
| | **Male(9)** | **Female(20)** |
| **Skin** | 8 | 18 |
| **Renal** | 3 | 5 |
| **CNS** | 1 | 8 |
| **CVS** | 1 | 1 |
| **Joint** | 9 | 19 |
| **GIT** | 2 | 10 |
| **RS** | 3 | 4 |

**Table-9**

**ANA positivity among males and females**

| Samples | Total number | ANA positivity | |
|---|---|---|---|
| | | Number | Percentage |
| **Males** | 32 | 9 | 28.12 |
| **Females** | 58 | 20 | 34.5 |

9 out of 32 male patients showed positive for the presence of anti-nuclear antibodies.

20 out of 58 female patients showed positive for the presence of anti-nuclear antibodies.

**Figure- 8**

**Distribution of ANA positivity among males and female**

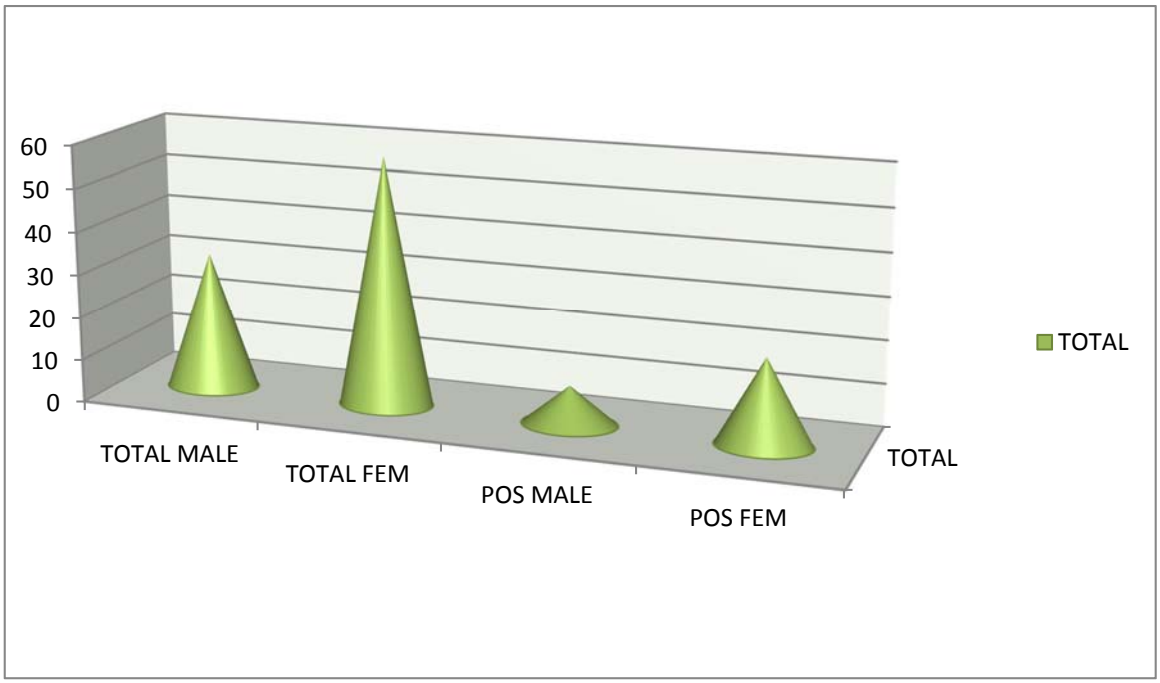


**Table-10**

**Age wise distribution of ANA positivity**

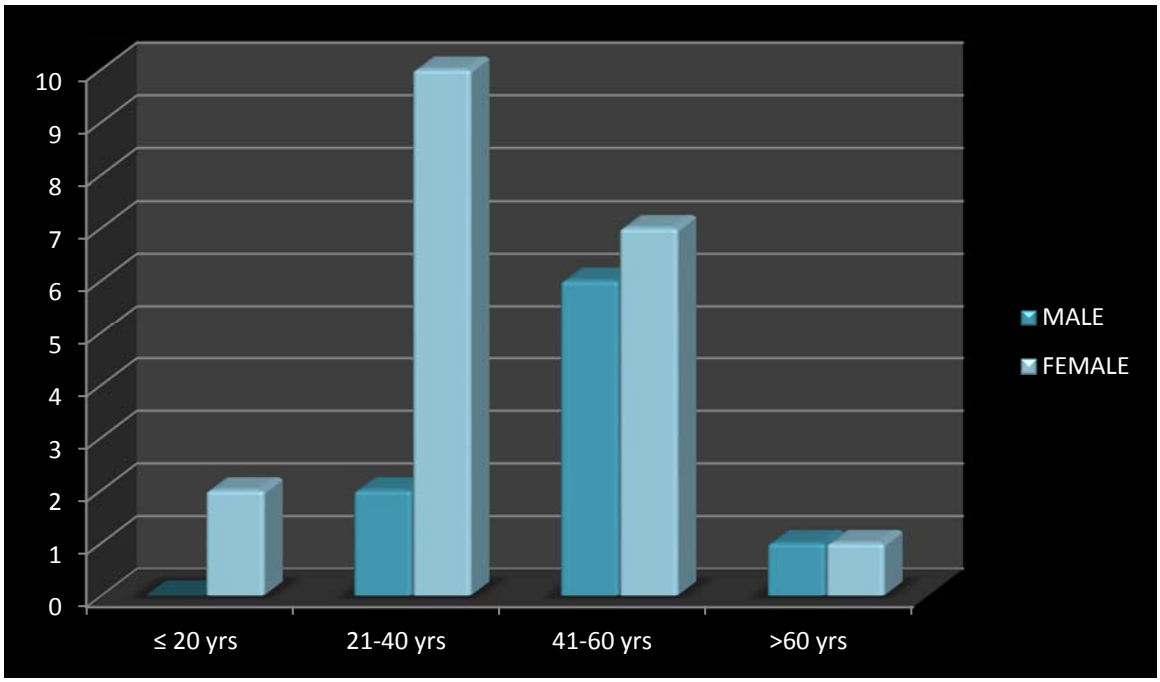
| Sl no | Age in yrs | ANA positive | Male | Female |
|---|---|---|---|---|
| **1.** | **≤ 20** | 2 | 0 | 2 |
| **2.** | **21-40** | 12 | 2 | 10 |
| **3.** | **41-60** | 13 | 6 | 7 |
| **4.** | **>60** | 2 | 1 | 1 |

Table-10 shows that samples reporting ANA positive is more in females during reproductive period (10) and post menopausal period (7). Extremes of age, autoimmune affection is low being 2 in less than or equal to 20 years as well as 1 in women greater than 60 years.

Males show a slight increase during 21-40 years of age (2). During the fourth and fifth decade there are more positives (6). Similar to females, males also reported very low samples in extremes of ages.

**Figure-9**

**Age wise distribution of positive ANA cases**




Bar diagram showing that CTD infections are seen in between 21-60 years. Beyond these range ,the possibility is low.

**Table-11**

**Distribution of HEp-2 cell pattern among IIF assay**

| Sl.No | HEp-2 cell pattern | No . of positives | Percentage positivity |
|---|---|---|---|
| **1.** | **Coarse speckled** | 12 | 46.2 |
| **2** | **Homogenous** | 5 | 19.3 |
| **3** | **Cytoplasmic** | 3 | 11.5 |
| **4** | **Membranous** | 3 | 11.5 |
| **5** | **Nucleolus** | 3 | 11.5 |

The above table shows distribution of HEp-2 cell pattern among 26 positives detected by indirect immunofluoresence microscope.

Coarse speckled pattern were seen in 12 samples(46.2%. this pattern constitutes nearly half of fluorescent images viewed.

This is followed by the homogenous pattern which is present in 5 samples representing (19.3%).

The other three patterns that include cytoplasmic, membranous and nucleolus were positive in 3 samples each. The positive percentage is 11.5% in the above three.

This pattern has been confirmed by two independent observers. There were no controversies and both interpretations coincides.

Other patterns like anti centromere , vimentin are not observed in these samples.

**Figure-10**

**Distributions of HEp-2 cell pattern among IIF positive cases.**

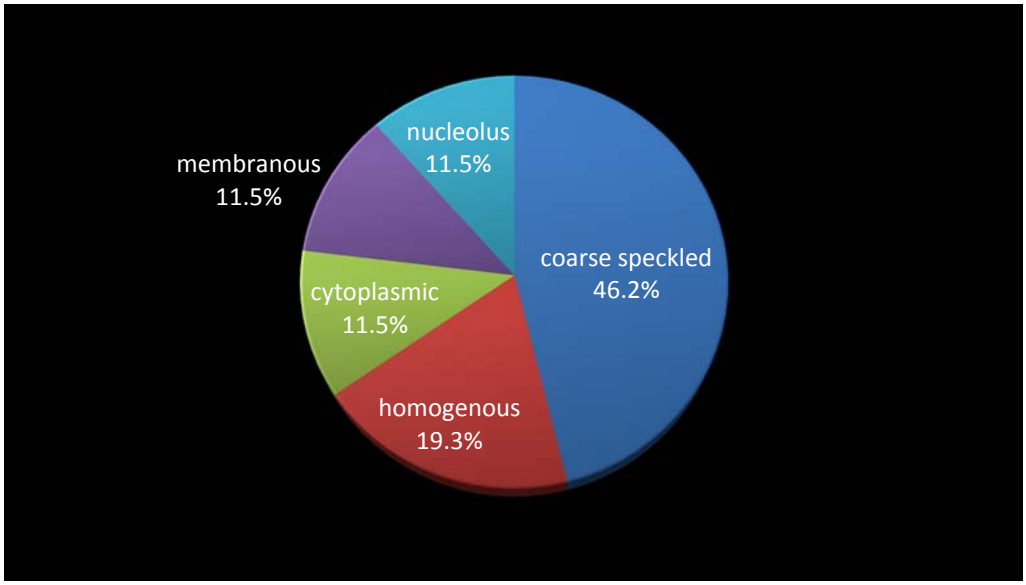



Figure-10 representing the various HEp-2 patterns obtained in fluorescent microscope**.** Coarse speckled seen in 46.2% of the samples.

**Table-12**

**Grading of fluorescent intensity among IIF assay positives**

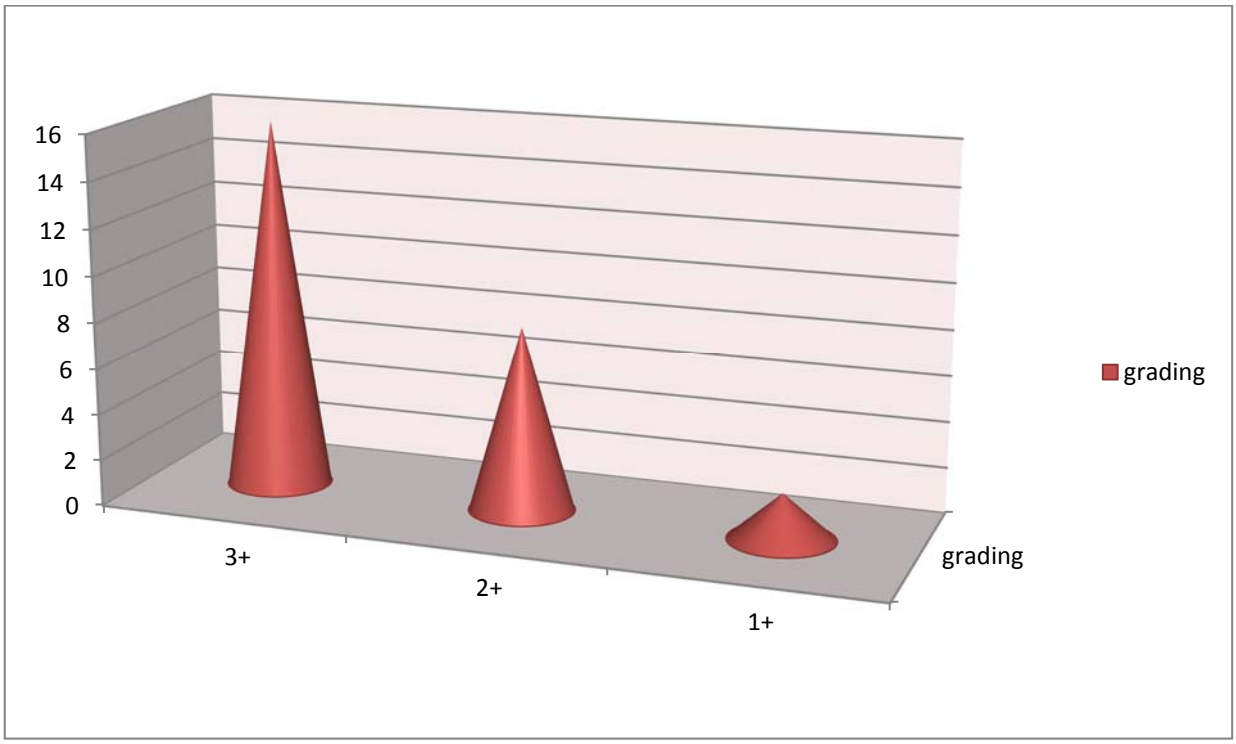
| Grading | No of positives | Positive percentage |
|---|---|---|
| **3+** | 16 | 61.6 |
| **2+** | 8 | 30.7 |

| | | |
|---|---|---|
| **1+** | 2 | 7.7 |

The intensity of fluorescent images were graded as 3+ ,2+,1+ .the table shows the various intensities of the HEp-2 pattern seen.

**Figure-11**

**Representation of grading of fluorescent patterns**




61.6% of the fluorescent images were in grade three +

30.7% of patterns were of two + grades..

7.7% showed the fluorescent images intensity as one+.

**Table – 13**

**Anti-nuclear antibodies detected by ELISA**

| Samples | ELISA positive | | ELISA negative | |
|---|---|---|---|---|
| | NO | % | NO | % |
| **Male** | 5 | 5.5% | 27 | 30% |
| **Female** | 15 | 16.7% | 43 | 47.8% |

Table -7 shows the ANA detected by ELISA. Out of the 90 samples 5 male Samples (5.5%) and 15 female samples (16.7%) are positive for ANA.

**Figure-12**

**ANA positivity based on ELISA**

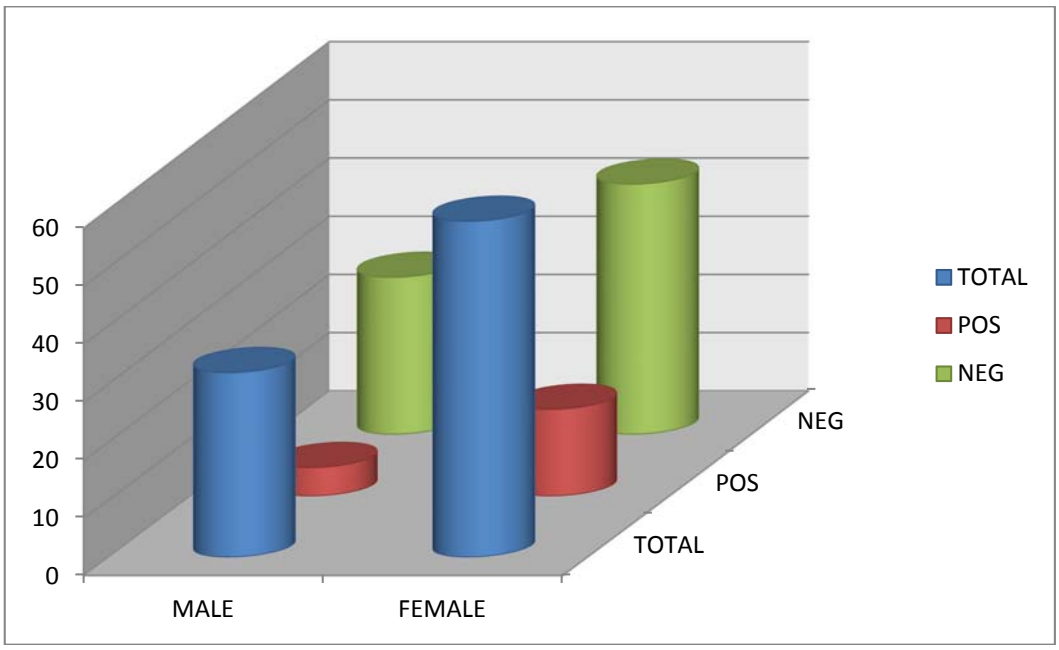



**Table-14**

**Anti-nuclear antibodies detected by IIF**

| Samples | IIF positive | | IIF negative | |
|---|---|---|---|---|
| | No | % | No | % |
| **Male** | 7 | 7.9% | 25 | 27.7% |
| **Female** | 19 | 21.1% | 39 | 43.3% |

The positivity rate among the screened samples is 7.9% for males and 21.1% for females respectively by immunofluorescence assay.

**Figure-13**

**Distribution of ANA positives by IIF.**

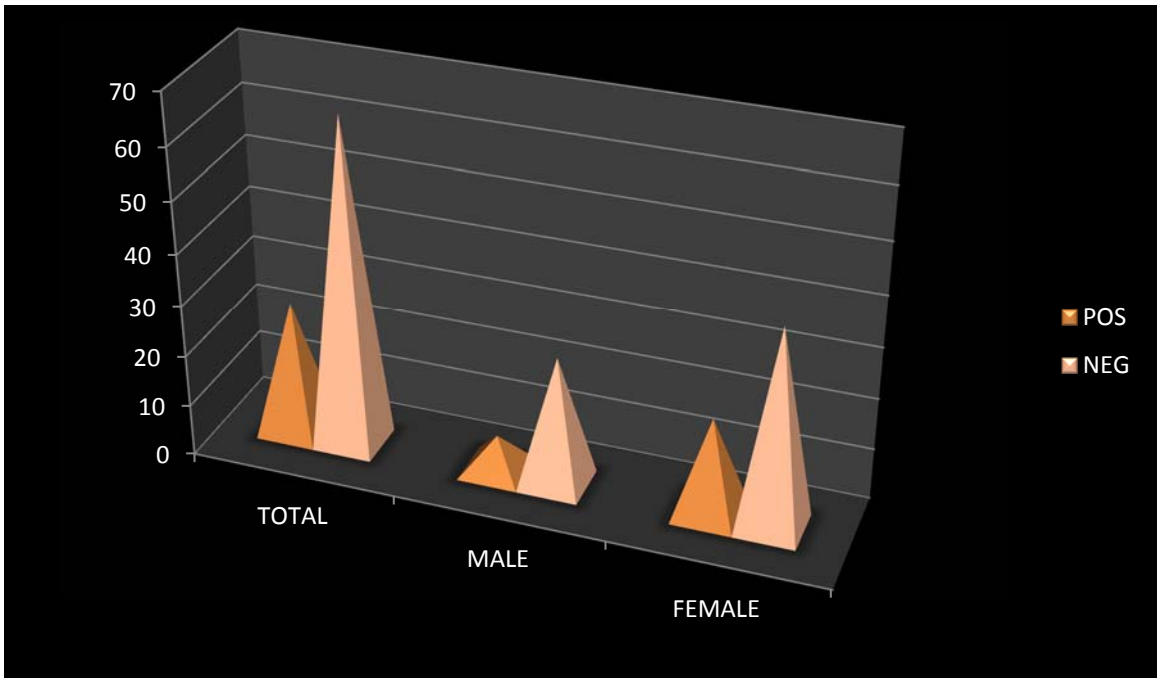



**Table -15**

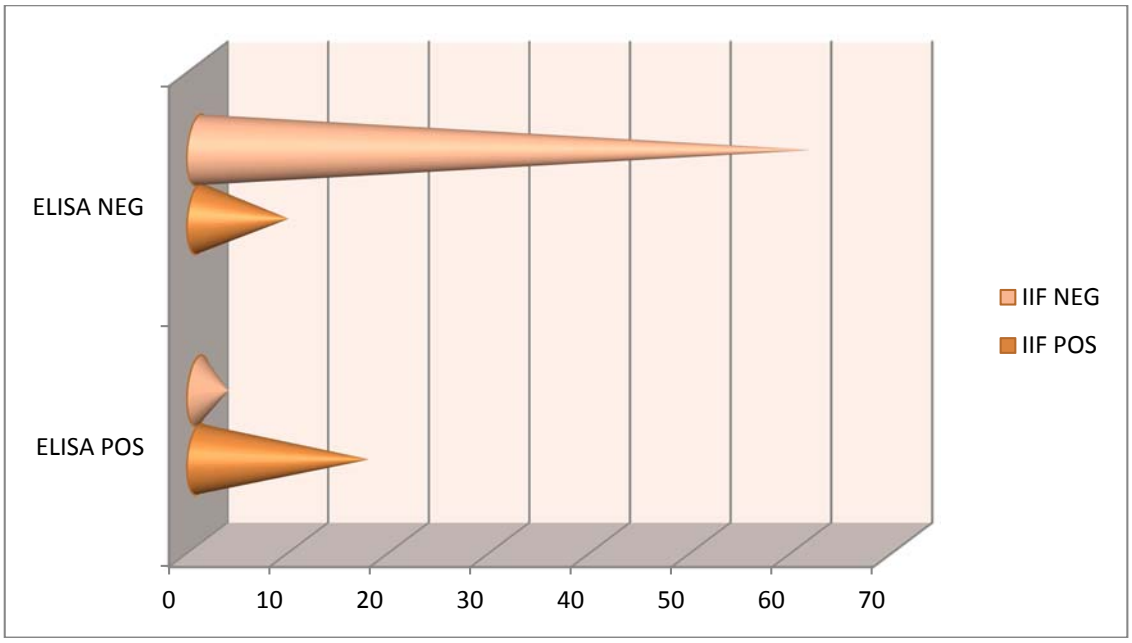
**Comparison of positivity between ELISA and IIF**

| ELISA | IIF | |
|---|---|---|
| | **Positive** | **Negative** |
| **Positive** | 17 | 3 |
| **Negative** | 9 | 61 |

In the above table, Enzyme immunoassay was evaluated against IIF. 17 cases showed positive for the presence of ANA by both IIF &ELISA. 3 of the cases positive by ELISA are negative by IIF and 9 cases positive by IIF are negative by ELISA.61 cases proved negative by both IIF & ELISA.

**Figure-14.**

**Comparison of ANA positives by IIF & ELISA.**




The McNemar p value is 0.146, which is not significant. This means the gold standard IFAT is better than ELISA.

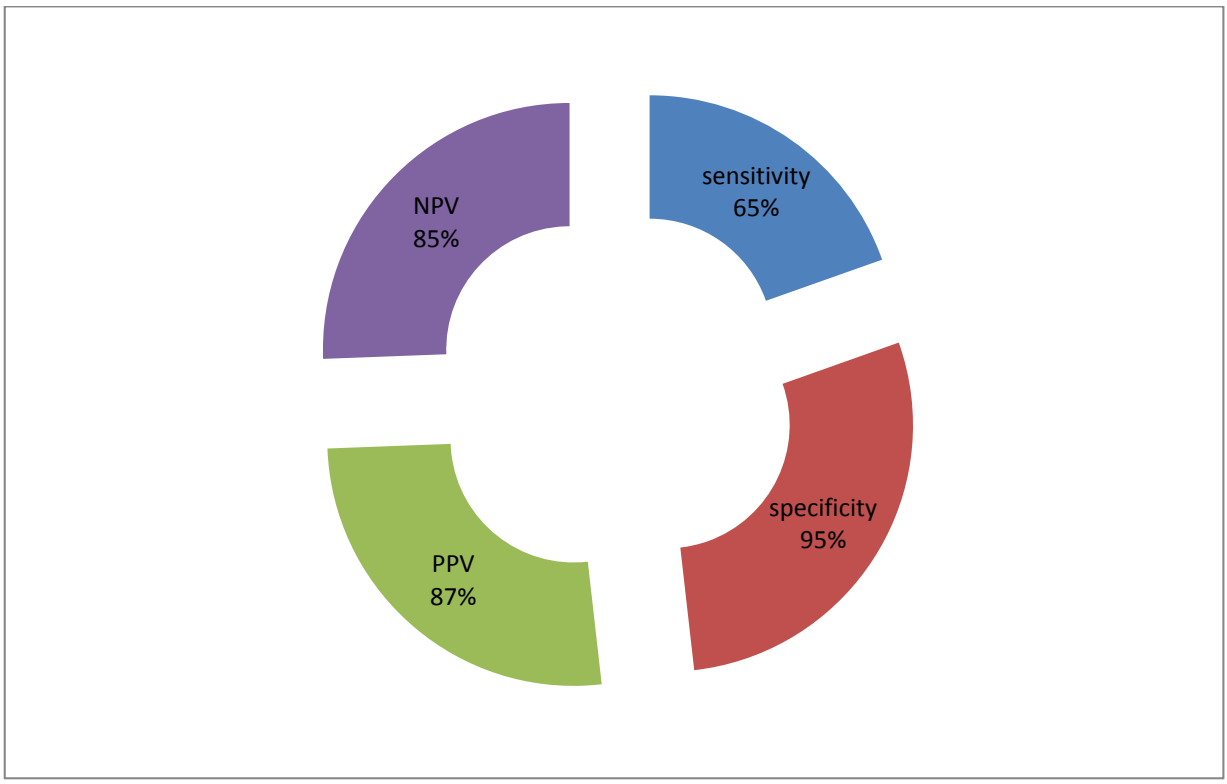
The Kappa agreement between the two methods is 65% and the Kappa p value is <0.0001, is significant.

Sensitivity which measures the true positives of the test and the specificity which measures the true negatives of the test are validated.

Positive predictive value (PPV) and the negative predictive value (NPV) of ELISA were assessed.

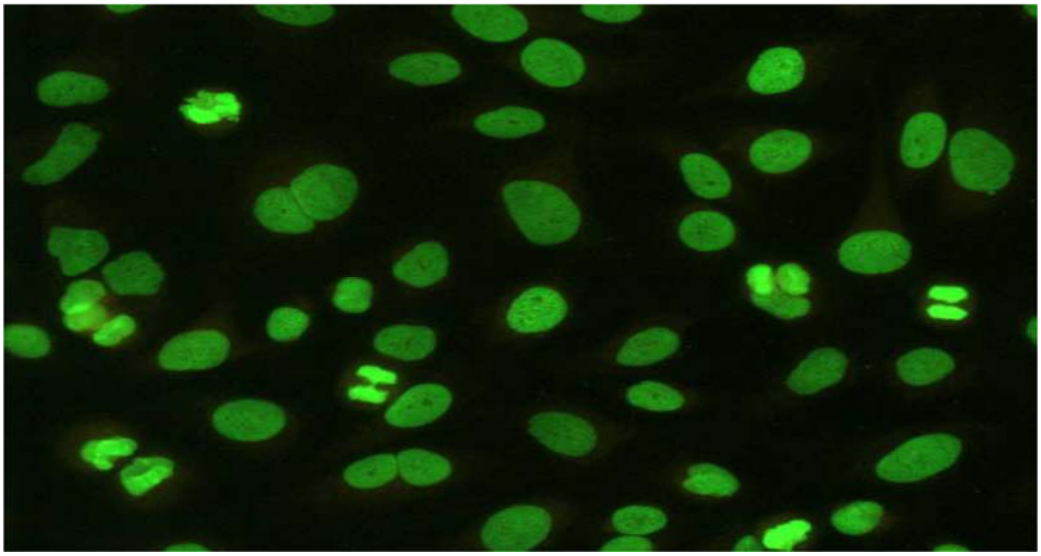
**Figure-15**

**Evaluation of ELISA for detecting ANA positivity among Connective Tissue Disorders.**

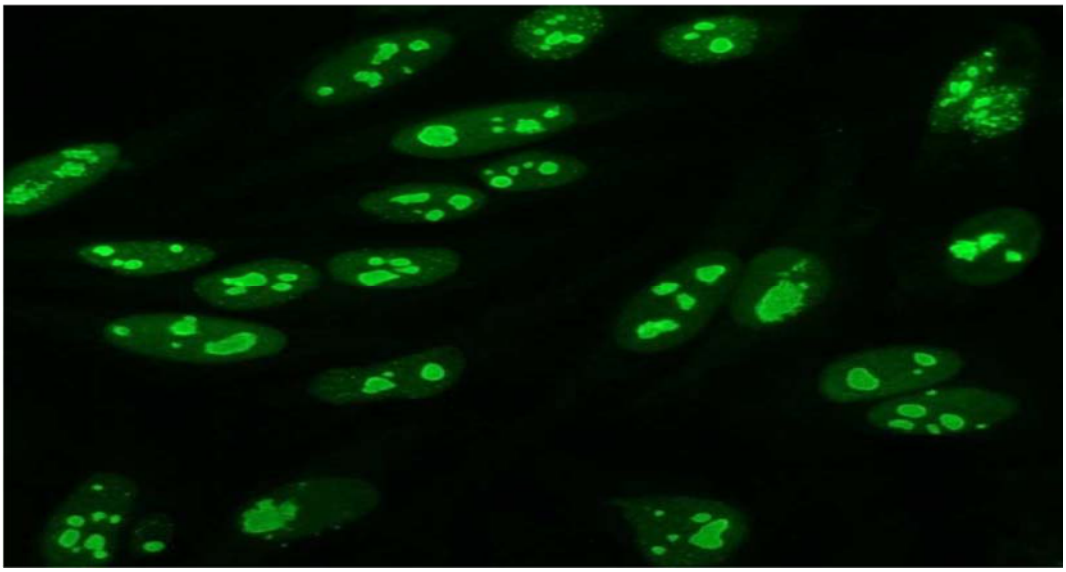



The above table shows sensitivity, specificity%, positive and negative predictive value of ELISA as 65%,95%,87% and 85% respectively.

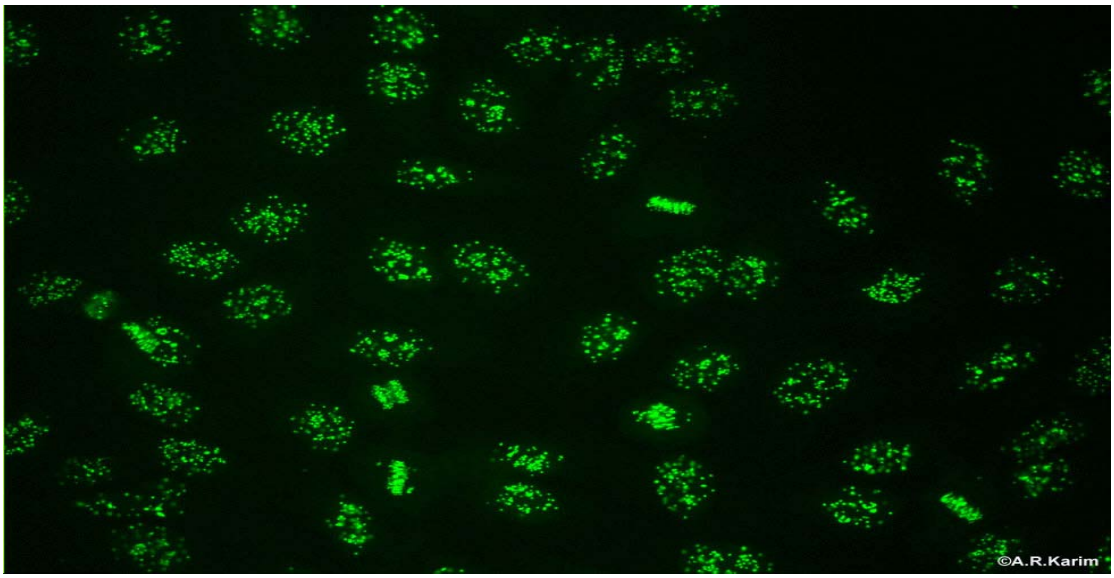
**HOMOGENOUS NUCLEAR PATTERN**



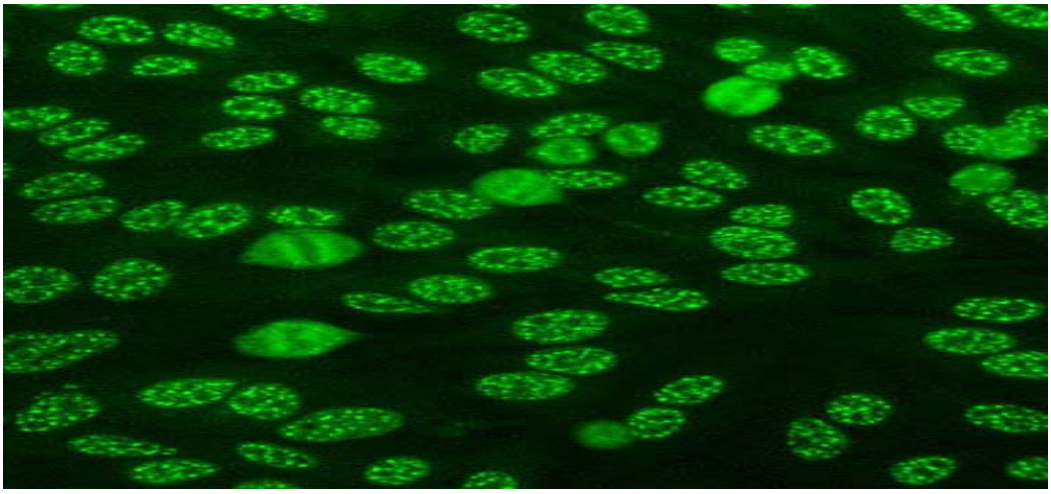
**NUCLEOLAR PATTERN**



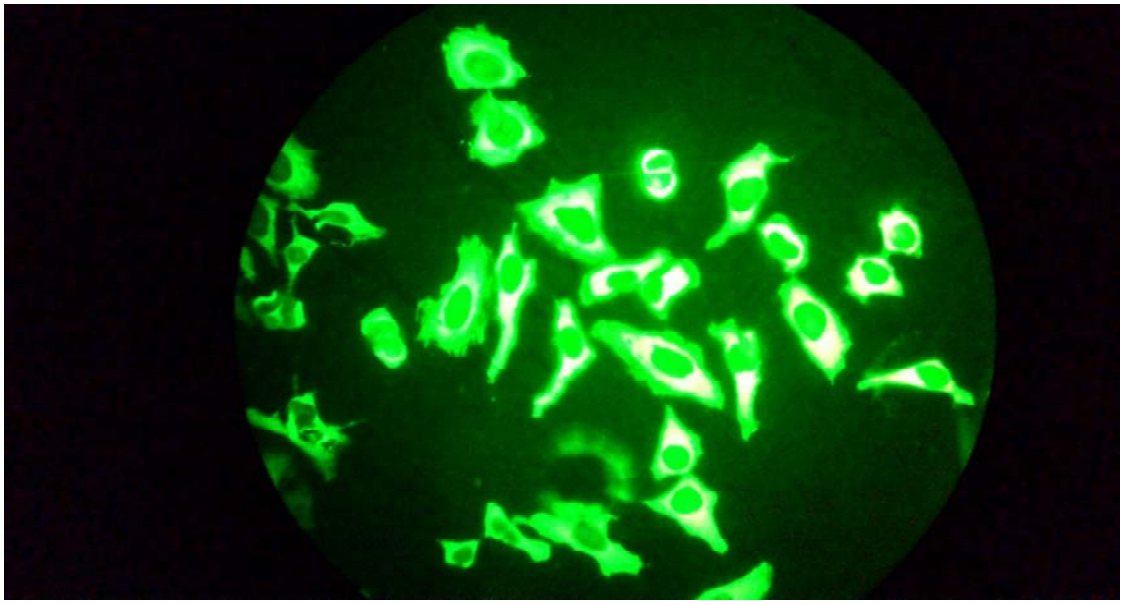
**CENTROMERE PATTERN**



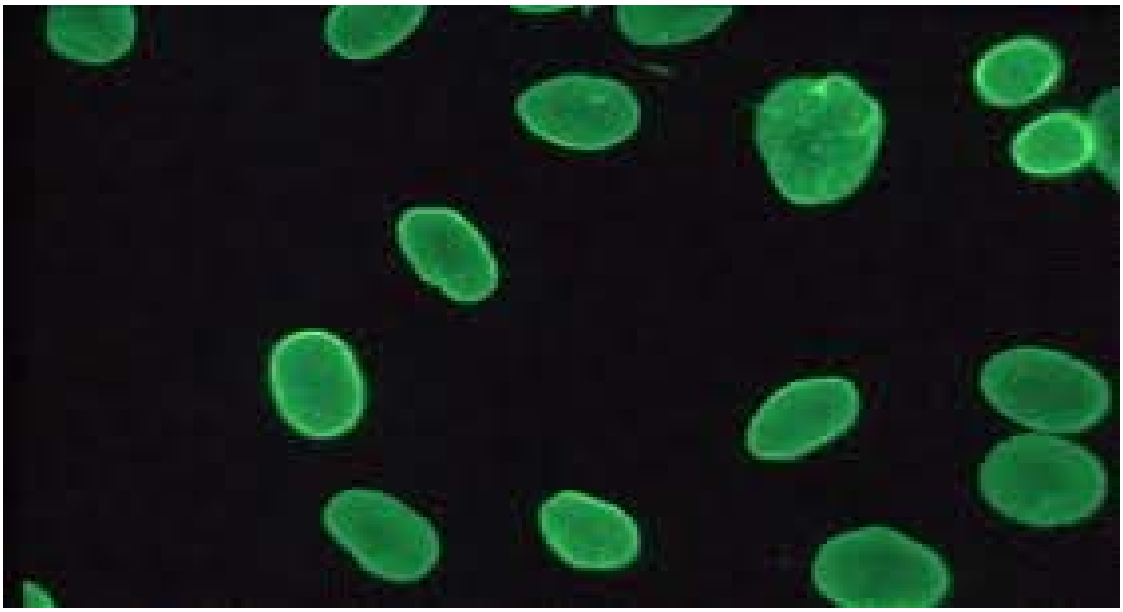

**SPECKLED NUCLEAR PATTERN**



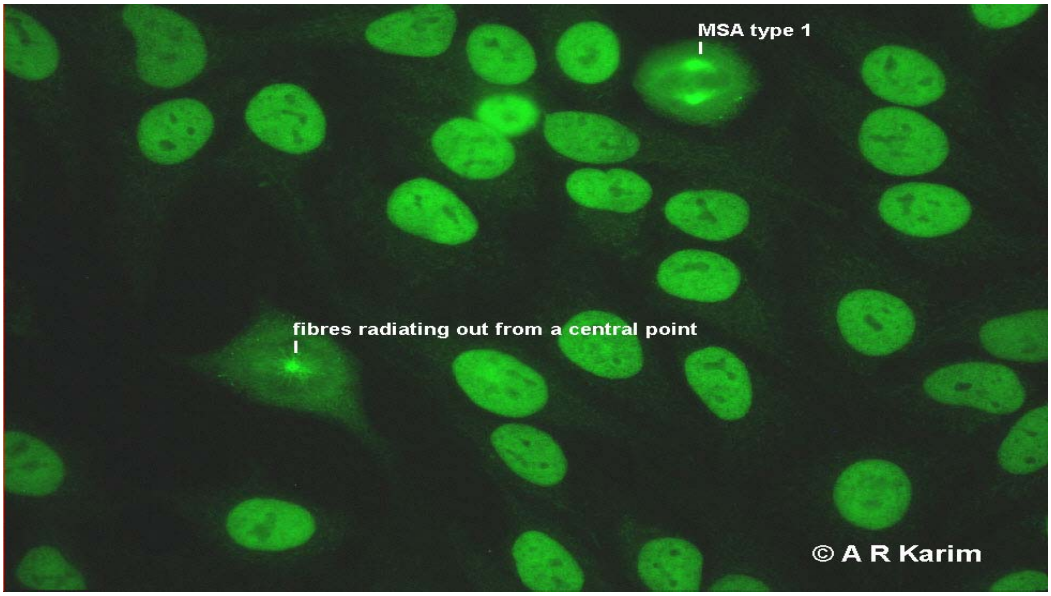
**CYTOPLASMIC PATTERN**

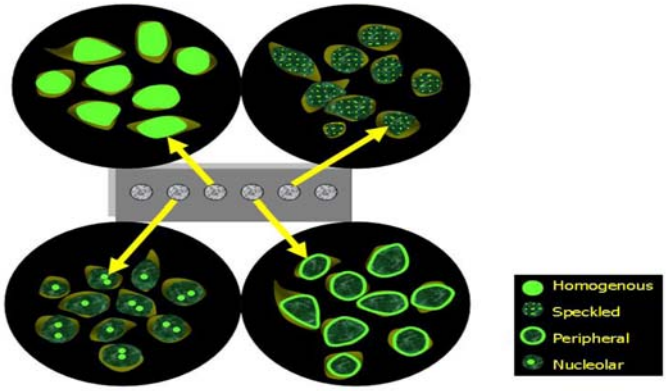


**MEMBRANOUS PATTERN**



**MIED PATTERN**


MSA type 1
fibres radiating out from a central point
© A R Karim


Homogenous
Speckled
Peripheral
Nucleolar

## DISCUSSION

Auto immune diseases have multifactorial etiology.CTD are a group of autoimmune diseases having a wide range of systemic manifestations. The breakdown of the normal immune homeostasis by several mechanisms was proposed for the genesis of the auto immune diseases.Anti-nuclear antibodies against the self-antigens are generated by the immune system that has lost its ability to maintain the immunological tolerance.

The study population mainly consists of clinically suspected CTD patients attending medicine clinics, nephro clinics and dermatology clinics, Tirunelveli Medical College Hospital, from February 2015- June 2015.

**6.1 Distribution of Clinical diagnosis among samples**

Among the samples, the provisional diagnosis arrived by the clinicians documented that Systemic Lupus Erythematous was the most commonly suspected CTD.40 samples (45%) were diagnosed as SLE. Our study is in accordance with the prevalence data of SLE both worldwide as well as in India.

12 samples (13%) were labeled as from Rheumatoid arthritis patients. This is followed by Dermatomyositis 4(4.5%), Mixed Connective Tissue Disorder 4 (4.5%), Scleroderma 3 (3%), and Sjogren's 2 (2%).

Around 25 samples were difficult to categorize and they were grouped as suspected CTDs. These suspected CTD constituted about 28% of the samples.

**6.2 Age and gender wise distribution**

In this study, most of the clinical samples were between 21-60years. The mean age of the patients is around 40 years. Females, particularly in the reproductive period 21-40 years (43.3%) are affected more than males (31.3%).

In this study antinuclear antibodies were found in 20 (34.5%) women and in 9 (28.12%) men.

Rus V in Denmark compared the positivity between males and females, found a higher fold positivity of fourteen. In a screening done among blood donors in Kulalampur, using IIF assay females show a rise in titer of 10.5% than males 5.4% ,the p value being >0.05.

In a study conducted in Salt Lake City in USA, among systemic rheumatic disorders of 100 samples, ANA was found to be positive in 37(37%) patients and negative in63(63%) patients. This is in close correlation with our study.

**6.3 Analysis of family history among CTD patients**

Out of the 90 samples, only 4 patients had a family history. Rest of these patients gave a negative family history. All these 4 patients proved positive for ANA in either of the methods. 25 patients with positive ANA in the assays did not have similar manifestations in the family.

Autoimmune diseases are as a result of many contributing factors. Family predisposition is one among them.

There is also a possibility of bias at the patient level since they may not be well aware of these symptoms among their family members at early stages.

**6.4 Seropositivity among females with bad obstetric history**

Antibodies produced against the nuclear antigens meddle with the effective placentogenesis. This also affects the placental maturation resulting in miscarriages. The effect of SLE on pregnancy is unpredictable as well as pregnancy has varied effects on SLE activity.

The auto antibodies belong to IgG class and hence they can cross the placenta.

In our study, among seropositive samples, 5 cases of BOH were positive for ANA, while 2 BOH were negative for ANA.

15 women, who documented no history of any miscarriages, were found to have auto antibodies.36 females were both negative to ANA and BOH.

Among seropositive for ANA, 25% reported positive for BOH; 75 % reported negative for BOH.

Up to 50% of the normal pregnancy may show positive for ANA, but will be of very low titers. Infections might be an inciting cause and need not be an auto immune.

Various studies had been put forth to find the association of ANA with pregnancy loss. Malinowski et al, done an ANA screening assay and arrived a

seropositivity of 51.5% in women with repeated miscarriages. This comes to 50% in ANA diagnostic procedure by Ticconi et al.

Nakatsuka M et al and Garcia et al studied independently and found antibodies are present in 43.5% &30% in habitual aborters. The control group in their study showed a prevalence of ANA as 22.4% and 6.6% respectively.

There was no association between the controls and women with pregnancy loss in the study designed by Bustos and also by Ruiz.

### 6.5 Seropositivity among samples

Among the samples diagnosed as SLE, 20 of them showed positive for ANA. In case of Dermatomyositis, Rheumatoid arthritis, Scleroderma, MTCD and suspected CTD, the positive samples were 3, 2, 2, 1&1 respectively.2 cases suspected as Sjogren's were negative for ANA. Studies are there to substantiate that SLE is the most common CTD[54].

Primary Systemic Sclerosis (Sjogren's) is very rare in India[66].This study also sounds same.

In our study, the Rheumatoid arthritis showed low prevalence. This might be attributed to decrease reporting by females in tertiary centers due to social circumstances or it might even be due to attending these hospitals at later stages of the diseases.

### 6.6 Distribution of manifestation among samples

In this study, both male and female had skin and joint manifestations in equal proportions. The involvement of gastro intestinal system and respiratory

system is high among females. The renal manifestation showed greater propensity towards male sex. Very few had central nervous system &cardiovascular involvement.

**6.7 Evaluation of manifestation among seropositivity**

It is documented in this study that renal involvement is seen in 3 patients out of 9 male positives (33.3%) and 5 patients out of 20 female positives (25%).

In an article submitted by Marten and Thomas from Lund University, male sex showed increased involvement of renal system. Even though in Anti – GBM or Good pasture's diseases, both sex are equally affected, IgA nephropathy and membranous nephropathy male to female ratio is 1-3:1 to 2:1, this correlates with our study.

M.Cojocaru, analyzed the lung involvement in various autoimmune diseases and reported SLE is the commonest among them to affect pleural cavity. In our study, among the 7 positives for ANA, 6 persons had been diagnosed as SLE.

**6.8 analyses of immunofluorescence and ELISA for detection of ANA**

In this study, ANA was screened by using IIF assay and ELISA.

IIF and ELISA are done frequently in country set up. Immunofluorescence uses substrate cells, the HEp-2 cells that have hegemony. Their origin is from human epithelioma type 2-clone CCI 23 ATCC and are cultured in monolayer, with a growing cycle of 36 hrs. Every phase of cell cycle can be visualized in the same slide.

In the IIF procedure, 7 males (7.9%) and 19 females (21.1%) were found positive for ANA. In the ELISA assay 5 males (5.5%) and 15 females (16.7%) were seropositive for ANA.

17 samples were positive for the antibodies and 61 samples did not show the presence of antibodies by both of the assays.

Discrepancies noted between 12 samples; 3 positive by ELISA were negative in IIF assay whereas IIF showed fluorescent patterns in 9 sera that were negative in ELISA report.

In this study, ELISA test was evaluated for its sensitivity and specificity against IIF assay. The sensitivity, specificity, positive predictive value and negative predictive value were 65%, 95%, 87% and 85%.

In a evaluation of a rheumatic disease conducted in Bangladesh by Dipti ANA positivity was found to be 100% in SLE. This is in acceptance with EL-Chennawai, Kumar Y & Gill MJ.

In various places, attempts were made to find a more suitable method to detect ANA among systemic rheumatic diseases. Positivity rate for IIF was 67.5% and for ELISA 27.5%. 5% showed negative by the above two procedures. This difference in positive rate is understood by the p value<0.0007.

Sebastian compared IIF with Line immunoassay and concluded IIF as a best screening method. Among the various fluorescent images interpreted

homogenous nuclear pattern in seen (45.5%) followed by speckled nuclear pattern (36%).

Priyadarshini et al found that ANA by ELISA is less sensitive than IIF using HEp-2 extract and it is about only 71.43%. The sensitivity is similar to our study. The specificity was around 86, 84%.She also observed that homogenous nuclear pattern is in half of the positive samples, followed by speckled. Sunitha et al reported cytoplasmic pattern to be more common than homogenous[100].

Chopra et al of India detected ANA and reported homogenous nuclear pattern to be common followed by speckled nuclear pattern. Similarly in a study interpreted by Ogaswarsa homogenous nuclear pattern (44.44%) followed by speckled nuclear pattern.

In our study speckled nuclear pattern is seen in 12sera (46.2%) was found to be common, followed by homogenous nuclear pattern 5 (19.3%). In a study, in Spain, speckled nuclear pattern was found to be 50%.

In a study conducted by Michael dense speckled pattern was found in 33.1% of ANA positive healthy individuals when compared to 0% of ANA positive SARD patients. It is better to consider that ANA may be present some few years before the symptoms arise.

In Japan Kumagai, evaluated the presence of ANA in known CTD patients with equal number of healthy volunteers by ELISA & IIF. The cut-off value which segregates the healthy controls from CTD patients was determined

using receiver operator characteristics (ROC) analysis and projected that Enzyme Linked Immunosorbent assay was better than manually visualized fluorescent patterns produced on HEp-2 substrate. But Gonzalez of Spain found no significant difference between ELISA &IIF using ROC analysis.

In a study conducted in Bangladesh, on CTD patients, by both methods ELISA &IIF, the sensitivity and specificity of ELISA were 90.7% & 85.7%.

The efficacy of five different types of commercial ELISA kits to detect ANA was done in comparison with IIF, represented as positivity of 92% by immunofluorescence assay and arrange of seropositivity from 74%-94% by ELISA. The entire homogenous and speckled pattern produced by specific auto anti-bodies are invariably identified by ELISA.

Bonilla et al, compared IIF microscopy with fluorescent beads and found the sensitivity of SLE as 90.6% than FB 49.1%,(p value<0.0001). in terms of specificity FB is superior to IIF. Shovman proposed that FB is superior to IIF in screening.

Jorn Voigt compared the visual IIF demonstration with automated interpretation of IIF and showed 272 positive results by both methods .in addition automated method detects 77 positive out of the 79 interpreted manually . Considering the economic factors in our country IIF can be used as the best screening and if need arises negative ANA may be retested.

In our study, The McNemar p value is 0.146, which is not significant. This means the gold standard IFAT is better than ELISA. The Kappa

agreement between the two methods is 65% and the Kappa p value is <0.0001, is significant

ANA detection by IIF is the gold standard. Only in this method, the fluorescent images noticed can point to the type of CTD, Hence IIF may be considered superior to EIA .As the interpretation of ANA is subjective, it is prone for inter –observer variability. The intensity of fluorescence representing the avidity of ANA to substrate cells and the level of auto anti-bodies may lead to inter-observer variability.

Moreover a plurality of fluorescent images is noted recently. This has been overcome by automated IIF evaluation. But the mixed patterns in IIF are a critical point to be considered and better software should be created to overcome this pattern differentiation.

IFAT shows positivity in low titers in few healthy patients. A negative result of ANA on HEp-2 assay is also noted in connective tissue diseases, probably due to the presence of very soluble antigens like antiSSA/Ro.

ELISA also shows high sensitivity and specificity, decreases the time to screen quite numerous sera at a same time. It is also easy to perform, no need for highly skilled staffs and can also be automated.

ELISA kits are also commercialized and their diagnostic utility demands some more studies to substantiate it.

An ideal technique should be highly sensitive and specific and both positive and negative predictive values should be correlated correctly .In addition it needs to be cost-effective, simple, quick to perform with limited technology. Unfortunately, none of the methods available till date satisfies the above said criteria.

**6.9 Guidelines to detect ANA**

1. ANA detection is useful in CTD

2.Isolated ANA positive has little value, hence the degree of clinical suspicion is very valuable

3.ANA presence do not confirm autoimmune disease, since literature points the association ofANA in healthy persons and in other diseases too.

4.Higher titers of ANA suggest a likelihood of rheumatic disorder ,but do not signifies diseases severity

5. negative ANA do not rule out the autoimmune diseases .some anti-Ro positive patients have negative ANA.

6. ANA levels fluctuate and so no need for repetition to monitor the diseases.

7. ENA show both diagnostic and prognostic value in SARD.

**6.10 In future!**

ANA detection has promising future era. The new technologies like multiplex immunoassays, antigen microarray provides a sensible alternative to

the traditional ELISA, immunoblot and IIF. There is vast improvement in the area of quantum dots and other fluorescent nanoparticles which will benefit the basic analysis in routine laboratory

## SUMMARY

This study was conducted from February 2015 to July 2015.The study population consisted of male and female patients attending medicine clinic, nephrology clinic and dermatology clinic, Tirunelveli Medical College Hospital, Tirunelveli.

- A total of ninety blood samples collected from clinically diagnosed & suspected Connective Tissue Disorders.
- The mean age among the samples is 40 years.
- Out of the 90 samples tested, 40 were suspected to suffer from Systemic Lupus Erythematous,2 from Sjogren's,3 from Scleroderma,12from Rheumatoid arthritis,4 from Dermatomyositis and 4 from Mixed Connective Tissue Disorder.

  About 25 patient were difficult to specifically diagnose and were categorized as suspected Connective Tissue Disorder.
- Of the total samples 32 were males and 58 were females.

- 9 (28.12%) of males and 20 (34.5%) of females showed positive for the presence of anti-nuclear antibodies.
- Of the total samples,5 (5.5%) of males and 15 (16.7%) of females found to be positive for anti-nuclear antibodies by Enzyme Linked Immuno Sorbent Assay.
- Among the samples,7 (7.9%) of males and 19 (21.1%) of females were found positive by Indirect Immunofluorescence Assay.
- The most frequent HEp-2 pattern observed in the study was coarse speckled followed by homogenous,cytoplasmic, membranous and nucleolar.
- Among the 26 samples found to be positive bt IFAT,3+ grading is seen in 16 (61.6%) samples, 2+ grading seen in 8 (36.7%) and 1+ grading is seen in 2 (7.7%) samples.
- The sensitivity and specificity of ELISA were 65% and 95% respectively. The positive predictive value was 87% and the negative predictive value was 85%.
- The McNemar p value is not significant indicating IFAT gold standard is better.
- There is Kappa agreement between the two methods is 65%.

## CONCLUSION

- The present study shows that auto antibodies play a role in Connective Tissue Disorders; and patients with suspected manifestations should be tested for antinuclear antibodies.
- Women are affected more than men.
- Indirect immunofluorescence is superior to ELISA in identifying antinuclear antibodies.
- Eventhough ELISA appears to be easy to perform with least interpersonal variations,the sensitivity depends on the auto antigens coated in the well. Commercial kits vary in this aspect.

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## ANNEXURE 1

**PROFORMA**

| NAME | AGE/SEX | IP/OP NO | OCCUPATION | ADDRESS |
|---|---|---|---|---|
| | | | | |

HISTORY

1. Cutaneous(skin) involvement

   Fever

   Macolopapular rash

   Skin thickening

   Other skin changes

2. CNS involvement

   Headache

   Vomiting

   Photophobia

   Seizure

5. Gastrointestinal involvement

   Constipation

   Diarrhea

   Heart burn

7. CVS (cardiac) involvement

   Chest pain

2.Renal involvement

   loin pain

   oliguria

   anuria

   haematuria

4. Bone (joint) involvement

   arthralgia

   early morning stiffness

   swelling

   numbness

6.Respiratory involvement

   cough

   dyspnoea

   wheeze

OBSTETRIC HISTORY

- Menstrual history
- LMP EDD GRAVIDA PARA
- LCB
- BOH, if any
- H/O contraceptive usage

H/O of family members with similar illness

H/O smoking/alcohol/drug intake

H/O HT/DM

General Examination

| | |
|---|---|
| Built | Temp |
| Pallor | Weight |
| Facial puffiness | Heart rate |
| Pedal edema | Respiratory rate |
| Cyanosis | Blood pressure |
| Jaundice | Abdominal distension |

INVESTIGATIONS

| | |
|---|---|
| 1. TC | 10.Urine : albumin/sugar/deposits |
| 2. DC | 11. Chest X ray |
| 3. Hb% | 12. USG/ECHO |
| 4. ESR | 13. CT SCAN |

5. Blood

- Sugar
- Urea
- Creatinine

14.CRP

15. ASO

16. RA

17.ANA

6. HIV status **ELISA**

7. HBsAg **IIF ASSSAY**

8. Thyroid profile

18. Skin biopsy

**CLINICAL DIAGNOSIS :**

**KEY TO MASTER CHART**

1. FH -Family History

2. DR -Duration of Illness

3. BOH -Bad Obstetric History

4. SKIN -Skin manifestations

5. RENAL-Renal manifestations

6. CNS -Central Nervous System manifestations

7. CVS -Cardio Vascular System manifestations

8. JOINT -Joint manifestations

9. GIT -Gastro Intestinal Tract manifestations

10. RS -Respiratory manifestations

11. ANA - Presence of Anti nuclear Antibody

12. ELISA - Results of ELISA

13. IIF -Results of IIF

14. MO - months

15. P/N -Manifestations present/absent

16. POS/NEG-Results positive/negative

17. NA - not applicable.